Investigating the growth and metabolic difference of Bvg+ and Bvg- phase Bordetella pertussis

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Abstract

Bordetella pertussis is the causative agent of whooping cough, which, despite the existence of an efficacious vaccine, continues to cause disease and death worldwide. The Bvg two-component system controls much of virulence and when Bvg is active virulence factors are expressed (Bvg+), and when it is not active they are not (Bvg-).

The Bvg regulon is large and much is still not understood about what it controls other than virulence. Spontaneous mutation of the Bvg system leads to the rise of Bvg- mutants, which out-compete other bacteria in culture, showing that the Bvg- phase *B. pertussis* has a growth advantage. The metabolism of Bvg+ *B. pertussis* is well characterised, but is not well understood in the Bvg- phase.

Through phenotypic assays it is shown that *B. pertussis* in the Bvg- phase grows and divides quicker, despite Bvg+ growth consuming more glutamate per gram of biomass and also generating a greater PMF. RNAseq reveals a difference in the way that glutamate is used as a carbon source as well as different expression levels of enzymes involved in the TCA cycle and electron transport chain. TraDIS shows that genes involved in elongation of the cell wall were essential for growth in the Bvg- phase.

These data point towards a model for growth whereby in the Bvg+ phase more glutamate is used in the TCA cycle for synthesis of branched-chain amino acids and reducing power, which is used in a electron transport chain to generate a greater PMF and more ATP. Cells in the Bvg- phase elongate faster to divide more often and there is a greater emphasis on synthesising aminosugars as peptidoglycan precursors, gluconeogenesis and maintaining pools of CoA.

Potential for using data gained about the growth and metabolism of the Bvg- phase to improve vaccine cultures are discussed.

List of Abbreviations

(p)ppGpp- Guanosine pentaphosphate
(q)PCR- (Quantitative) Polymerase chain reaction
ATP- Adenosine triphosphate
BG- Bordet Gengou
c-di-GMP- Cyclic di-guanylate
cAMP- Cyclic adenosine monophosphate
CCCP- Carbonyl cyanide 3-chlorophenylhydrazone
cDNA- Complementary DNA
CFU- Colony-forming unit
CoA- Coenzyme A
$DiOC_2(3)$- Carbocyanine dye 3,3'-diethyloxacarbocyanine iodide
DNA- Deoxyribonucleic acid
FHA- Filamentous haemagglutinin
GTP- Guanosine triphosphate
IFN-ϒ- Interferon gamma
IgG- Immunoglobulin G
IL- Interleukin
LPS- Lipopolysaccharide
MCS- Multiple cloning site
MIC- Minimum inhibitory concentration
mRNA- Messenger ribonucleic acid
$NADH/NAD^+$- Nicotinaminde adenine dinucleotide
NTPs- Nucleoside triphosphate
$OD_{(600)}$- Optical density (at 600nm)
PBP- Penicillin-binding protein
PBS- Phosphate buffered saline
PHB- Polyhydroxybutyrate
PMF- Proton motive force
Prn- Pertactin
PT- Pertussis toxin
RNA- Ribonucleic acid
RNAseq- RNA sequencing
SS- Stainer Scholte
T3SS- Type-three secretion system
TCA cycle- Citric acid cycle
TCT- Tracael cytotoxin
TEM- Transmission electron microscopy
Th1/2/17- T-helper 1/2/17
Tnseq- Transposon sequencing
TraDIS- Transposon-Directed Insertion Sequencing
β-HB- Beta-hydroxybutyrate

Chapter 1- Introduction

1.1 The classical *Bordetella* species

1.1.1 The classical Bordetellae

Bordetella pertussis is the causative agent of whooping cough, or pertussis, a respiratory disease of humans. There are nine species of *Bordetella* identified, although the ones most closely associated with human disease are the so-called "classical" bordetellae; *B. bronchiseptica*, *B. parapertussis* and *B. pertussis,* which have been shown by genome sequencing to share a common ancestor [1].

B. bronchiseptica infects a wide-range of mammalian hosts, typically in a chronic fashion, causing respiratory diseases including kennel cough in dogs. The bacteria can cause disease in humans, particularly in patients who are immunocompromised [2, 3]. *B. parapertussis* causes a pertussis-like disease in humans and may account for more cases of whooping cough than currently appreciated [4]. *B. pertussis* in considered a human-adapted lineage of *B. bronchiseptica* and circulates exclusively in humans. It was first isolated by Bordet and Gengou in 1906 from the sputum of a patient with pertussis [5], and work began to control the disease with the development of the first whole-cell vaccines in the first half of the 20th century.

1.1.2 Whooping cough

Pertussis disease, or whooping cough, is first thought to have occurred as an epidemic in the 16th century [6]. The disease was described as affecting children typically less than a year old, causing them to cough sometimes for 4-5 hours at a time. The cough was so severe that it prohibited flow of air through the airways causing a very characteristic sound to be made, called a "whoop". This cough was so violent that bleeding and vomiting occurred and even death, through the lung pathology produced.

Despite available vaccines and good coverage *B. pertussis* continues to be endemic and to cause disease all over the world. Typically the disease affects young children, pre-immunisation, and lasts for 6-12 weeks. Initially symptoms are similar to mild viral infections, with mild cough, but within 1-2 weeks the frequency and intensity of cough increases. The disease is characterised by lack of fever, standing it

apart from other respiratory illnesses, while leukocytosis occurs. The "whoop" sound is caused by effort to breathe at the end of a coughing fit, and as described in the 16th century vomiting is common. Disease can lead to pneumonia, which can be due to a secondary infection, or seizures and encephalopathy, which are due to the severe coughing fits. Internal bleeding can also occur. This stage of severe disease can last for 2-8 weeks before the coughing fits gradually decrease in frequency [7]. Although disease affects both adults and infants, severe disease and death are more common in infants. Severe leukocytosis is linked to pulmonary hypertension, which is a common cause of death from pertussis [8, 9].

1.2 Pathogenesis of *B. pertussis*

1.2.1 Adhesins

The bacterial factors driving the virulence of *B. pertussis* are well characterised. The bacteria express adhesins, such as FHA, coded for by *fhaB*. A large protein, FHA is 220kDa in its mature form [10]. Mature FHA binds epithelial cells via a Arg-Gly-Asp triplet in the middle of the protein [11], and stimulates an immune response, including antibodies [12-14]. *B. pertussis* expresses fimbriae, coded for by the genes *fim2* and *fim3,* and *fimX,* the latter if expressed at all is expressed at low levels [15-17]. All three genes are subject to phase variation due to slip strand mispairing induced expansion and retraction within a poly-C tract in their promoters, so the genes can be expressed, or not, independently of each other [18]. A common element of all three fimbrial types is FimD, which forms the tip of each of the fimbriae [19]. The fimbriae of *B. pertussis* have been shown to have a role in colonisation, specifically binding to monocytes and sugars present in the epithelium [20-22], and they also stimulate a protective immune response [23].

Pertactin (Prn) is an autotransporter 69kDa in size, which is thought to play a role in attachment, as the protein contains the same Arg-Gly-Asp triplet as FHA [24-26]. Antibodies are raised against this protein and are expected to provide protection, by preventing attachment, and encouraging phagocytosis [23, 27, 28]. Other autotransporters include tracheal colonisation factor (TcfA), BrkA and Vag8, thought to play various roles including colonisation, adherence and serum resistance [29, 30].

1.2.2 Toxins

There are several toxins expressed by *B. pertussis* that contribute to disease. One of these is adenylate cyclase (CyaA), which has haemolytic activity [31]. The toxin binds and enters target cells, where is it stimulated by calmodulin leading to the overproduction of cAMP [32]. The result is the inhibition of immune responses including chemotaxis and phagocytosis [33, 34]. CyaA also stimulates an antibody response [35].

Another toxin, dermonecrotic toxin, is an AB type toxin, with a receptor domain and an enzymatic domain. Once inside the cell the toxin stimulates activity of the Rho GTPase, causing remodelling of the cell and stimulation of DNA replication [36, 37]. The role of this toxin in pathogenesis of pertussis disease is not clear, since mutants unable to express the protein have no reduction in ability to cause lethal infection in a mouse model [38].

Trachael cytotoxin (TCT) is a subunit of peptidoglycan, produced by all Gram-negative bacteria and transported by AmpG from the cell wall into the cytoplasm as part of the normal recycling of the peptidoglycan wall [39, 40]. *B. pertussis* AmpG has reduced activity and therefore releases TCT into the environment. The TCT is sufficient and required to cause the characteristic pathology to ciliated epithelial cells seen in pertussis disease, thought to be caused by nitric oxide radicals produced via IL-1α secretion stimulated by TCT [41-43].

Pertussis toxin (PT) is the only toxin secreted exclusively by *B. pertussis*. It is an AB type toxin, made up of six peptide chains coded for by *ptxA-E*. *ptxA* codes for the enzymatic "A" domain, while the receptor "B" domain is made up of five peptide chains, the products of *ptxB-E* in the ratio 1:1:2:1 [44, 45]. The toxin is secreted from the bacteria via the Ptl apparatus, a type-IV secretion system (T4SS), made up of the products of nine genes within the same operon as the *ptx* genes [46, 47]. The B domain of the toxin binds receptors on host cells and gains entry to the cell, while the A domain has ADP-ribosylating activity, activating G-proteins in the eukaryotic cell membrane [48]. The result of this is the inhibition of adenylyl cyclase, activation of potassium channels and inactivation of calcium channels. The effects of PT include enhanced insulin secretion, sensitivity to histamine, and immune modulation [49, 50]. PT is the cause of leukocytosis seen in infections, which is a greater influx of leukocytes, including neutrophils and lymphocytes, into the blood [51-53]. PT has been thought of as the primary virulence factor of *B. pertussis*, although *B.*

parapertussis, which causes a pertussis-like disease (with the absence of leukocytosis), does not express PT [54, 55]. Therefore, although PT does contribute to the severity of disease during infections, it would be naïve to say it is the primary contributor, and does not directly cause the characteristic whooping cough.

1.2.3 Other virulence factors

B. pertussis possesses genes for a type-three secretion system (T3SS), which are thought to be fully transcribed, translated and yet don't lead to cytotoxicity in mammalian cells as the T3SS in other *Bordetella spp.,* leading to the conclusion that the system might not be expressed, but subject to post-translational control [56]. It was later shown that *B. pertussis* does express a T3SS system and secretes effectors that play a role in colonisation and immune evasion. Specifically, the T3SS enhances binding to macrophages and the respiratory tract, as well as supressing the innate immune responses to *B. pertussis* as well as the Th1, Th17 and antibody responses [57].

B. pertussis produces lipopolysaccharide (LPS), shown to exhibit toxicity to host cells [58, 59]. The LPS of *B. pertussis* exhibits many differences to that of other *Bordetella spp.,* one of which is the lack of an O-antigen, which plays a part in resistance to killing by complement. Thus *B. pertussis* is thought to induce serum resistance by expressing LPS as well as the autotransporter BrkA [60-62].

1.3 Vaccines for *B. pertussis*

1.3.1 Whole-cell pertussis vaccines

Work on making a vaccine against *B. pertussis* began soon after the bacterium was first isolated and characterised. These vaccines consisted of killed whole bacterial cells. A mouse model was developed to assess the protective effect of vaccine candidates [63] and a vaccine was introduced in the US during the 1940s, in combination with toxins from diphtheria and tetanus (DTP vaccine). Routine immunisation began in England and Wales in 1957 and the number of notifications for pertussis disease began to fall significantly [64].

The whole-cell pertussis vaccine was introduced across the developed world and uptake coincided with extreme drops in incidences of pertussis disease. However, there were still concerns. In the UK, reports that efficacy of early whole-cell pertussis vaccines could have been as low as 20% led to a change in the dose of the vaccine [65] and in Sweden an increase in incidence of pertussis disease in the 1970s led to questions about the efficacy of the vaccine and the cessation of vaccination in 1979 [66]. Efficacy studies on the whole-cell pertussis vaccines are summarised in [65] where the question of what is actually meant by protection is raised. It is suggested that protection from disease in vaccinated individuals may be greater than protection from infection, perhaps providing an insight into the resurgence in pertussis disease since vaccinated individuals may still be able to be colonised. It was also suggested that discrepancies in efficacy estimates between studies might be due to differences between the antigenic composition of the vaccine and that of the circulating strains. Various studies put efficacy of the whole-cell vaccine at 64-96%. Efficacy was also positively correlated with the number of vaccine doses given [67-69].

Despite the good efficacy of the vaccine, there were questions being asked about the safety profile. As far back as 1974 a causal relationship was established between receiving the vaccine and neurological complications in children within 24 hours of receiving the vaccine [70]. The benefits of preventing the disease were largely considered to out way the risks associated with vaccination, however, the risks of adverse effects to vaccinated children were well founded [71, 72].

By 1994 resurgence of pertussis disease had already been documented in well immunised populations in the US, probably due to waning of vaccine-induced immunity [73]. However, during the 1990s in England and Wales incidences of pertussis disease continued to fall, with 3-4 year cycles of peak disease [74]. Due to fears of resurgence an acellular pertussis booster vaccine was introduced for pre-school children in England in 2001. An acellular vaccine was used due the risks of adverse reaction associated with the whole-cell vaccine [75].

1.3.2 Acellular pertussis vaccines

Acellular pertussis vaccines consist of purified antigens from *B. pertussis*. It was demonstrated during the early 1990s that acellular vaccines had good efficacy, even 1-component vaccines consisting of purified, inactivated PT. Generally, it was shown

that the more components added to the vaccine, with FHA in a 2-component, or with FHA, Prn, Fim2 and Fim3 in a 5-component vaccine, the better the efficacy [76]. Since 2004 the UK has used an acellular vaccine, replacing the whole-cell due to the good efficacy of the acellular and the concerns of adverse effects associated with the whole-cell.

PT has long been considered one of the most important antigens of *B. pertussis*, and is released into the supernatant during growth, along with FHA, making purification relatively easy [77]. An acellular vaccine was first described and defined in Japan, and has been given routinely in the country since 1981. Pertussis disease has since been controlled in Japan. The vaccine was made up of 2 components, containing purified PT and FHA [78]. Acellular pertussis vaccines vary in components used and amounts. A study of 13 difference acellular vaccines has revealed less reactivity to the vaccines compared to the whole-cell vaccines, including less swelling and pain [79, 80].

The efficacy of the Japanese 2-component acellular vaccine was calculated at around 78-92% [81]. Further studies in Sweden in the 1980s, vaccination to pertussis having ceased in that country at that time, calculated the efficacy of PT 1-component vaccine at 32-54% and the 2-component vaccine at 45-69% [23, 82]. In summary the acellular vaccine has been shown to have similar efficacy to that of the whole-cell vaccine, and the more components used the better the efficacy [83]. The majority of the developed world now uses acellular vaccines to protect against pertussis, while whole-cell vaccines are still routinely used in the developing world. An adolescent booster is now recommended in addition to infant immunisations [84, 85] while, a maternal vaccine strategy was implemented against pertussis in the UK in 2012 with the aim of protecting infants during the period before first immunisation [86].

1.3.3 Immunology and effectiveness of pertussis vaccines

Antibodies are induced to all antigens included in the acellular vaccine, but it has been shown that titres decrease to a level beneath the limit of detection as early as fifteen months after the primary dose [87]. There has been an observed difference in the type of immunity provided between the acellular and whole-cell vaccines, with activation of T-cells playing an important part in immunity. Specifically, vaccination with whole-cell pertussis induces a Th1 response characterised by secretion of IFN-γ,

while vaccination with an acellular vaccine induced a more mixed Th1/Th2 response characterised by secretion of IFN-γ in addition to IL-5 [88]. It was noted that the immunity induced by the whole-cell vaccine was closer to that induced by natural infection, and that a Th1 response was essential to activate killing of intracellular bacteria. A role for a Th2 response was not ruled out, and it was suggested that antibodies induced by Th2 response could be useful in inducing killing through opsonisation. Furthermore, active PT and LPS has been shown to provoke a Th1 response. Such antigens are present in the whole-cell vaccine but not the acellular (which contains inactive PT) but are known to cause adverse effects [89, 90].

Studies have further compared long-term immune responses to immunisation by the acellular vaccine and natural infection with *B. pertussis*. After 5 years it was found that in both cases large IgG titres to pertussis antigens and specific T-cell responses were only found in a minority of subjects. Furthermore, while natural infection induces a Th1 response characterised by IFN-γ with no IL-5, the acellular vaccine induces more of a Th2 response characterised by IL-5 with little or no IFN-γ [91]. This provided evidence that the response to acellular vaccine was different to that provoked by the whole-cell vaccine, which was more similar to the response induced by natural infection.

The difference in the type of response provoked by each of the vaccines is borne out in practice and it has been shown that people who had only ever received the whole-cell vaccine were more protected from pertussis disease than people who had received doses of the acellular. People who had only ever received the acellular vaccine were at significantly more risk of disease, while this risk increase was increased slightly by a sixth does of the acelullar vaccine [92]. This again shows the decreased effectiveness of the acellular vaccine compared to the whole-cell in protecting from pertussis disease long term.

The problems with vaccines to pertussis in not providing long-lasting protection stems from waning of immune protection to natural infection. The whole-cell vaccine, while providing good protection, does not protect for life, and the acellular vaccine containing fewer antigens stimulates a different type of immune response and protection wanes faster [93]. In a study conducted in the US it was estimated that efficacy in preventing pertussis decreased from 75% to 41% 1-2 years post-vaccination [94].

The problems with the acellular vaccine do not end with the waning of immunity. In one study infant baboons were subject to vaccination regimes with either the whole-cell or acellular vaccine [95]. While the acellular vaccine did protect against disease, it did not provide protection to infection and these vaccinated subjects were able to transmit the bacteria. Furthermore, the subjects vaccinated with the whole-cell vaccine, or who had previously suffered pertussis disease cleared reinfection more quickly than those vaccinated with the acellular vaccine. Differences were seen in the immune response in different cohorts. All subjects showed a robust antibody response, and while the subjects that were naturally infected showed a strong Th1 and Th17 memory response, only a weaker Th1 response was shown in subjects vaccinated with the whole-cell vaccine. Those vaccinated with the acellular vaccine showed a Th2 response, an even weaker Th1 response and no Th17 response. Furthermore, a mucosal immune response characterised by Il-17 seems important to induce robust immunological memory to *B. pertussis*.

Thus while the acellular vaccine is efficacious and provides as good protection as the whole-cell from pertussis disease in the short term, with less adverse reaction, immunity wanes quicker and the vaccine fails to protect against colonisation or transmission. This explains the ability of the acellular vaccine to control whooping cough but waning immunity and continued circulation of the bacterium leaves the population open to resurgence.

1.4 Epidemiology of pertussis and resurgence

1.4.1 Current epidemiology of *B. pertussis*

Despite good vaccine coverage, *B. pertussis* continues to circulate and cause disease worldwide [96]. Part of the reason for this is the waning immunity provided by the acellular vaccine. It has been estimated that even in areas with good vaccine coverage, the proportion of susceptible children becoming in infected is 60% within 5 years of vaccination, and 100% within 15 year, demonstrating the lack of long-term protection provided [97].

Epidemics of pertussis are cyclic, as they were before vaccination, although reduced in magnitude. However, it has been suggested that the rate of pertussis

disease in the US is still around 370-1500 per 100,000 and the rates of infection much higher, leading to between 800,000 and 3.3 million cases of disease per year [98].

There has been a change in epidemiology, with a shift in disease burden to adolescents and adults. In the US in the epidemic years 2004 and 2005 adolescents represented 36% and 30% of total disease cases [99]. During epidemics high disease rate in areas with high vaccine coverage among fully vaccinate groups indicates problems with vaccine failure. Most countries, including those that vaccinate with the whole-cell vaccine only, are experiencing a rise in cases of disease [99].

1.4.2 Resurgence of pertussis

While the resurgence of pertussis was noticed in North America by the early 1990s [73], it was not yet seen in Western Europe, however, by the early 2000s it was clear that pertussis rates were increasing. Indeed for the period 1998-2002 rates among adults doubled [100].

It was suggested that the 2012 epidemic in the US was the largest for 50 years [101] though part of the reason suggested was increased awareness and better diagnosis. The advent of PCR-based diagnosis techniques in the 2000s made for better diagnoses and the uncovering of an apparently previously underreported disease. However, the switch from whole-cell to the acellular vaccine providing protection for a shorter period of time was cited as a contributor [101].

That resurgence is seen worldwide, even in countries that vaccinate with the whole-cell vaccine, suggests that the problem lies not just simply with the acellular vaccine per se, but failure of either vaccine to provide long-lasting immunity or control transmission. Evolution of *B. pertussis* itself, thus escaping either vaccine, has been suggested as another cause [102]. The UK saw an outbreak in 2012 leading to over 9000 laboratory-confirmed cases and 14 infant deaths [103]. The switch to acellular vaccines, improvement in diagnostics and evolution of *B. pertussis* itself were seen as causes.

1.4.3 Adaptation of *B. pertussis* to escape vaccine control

It has been shown that *B. pertussis* is evolving in response to vaccination. A study from the Netherlands showed that there are variations in antigens between strains used to make vaccines and circulating strains [104]. This could simply be due to

natural antigenic drift, although particular notice was given to variation in vaccine antigens Prn and PT and the variation in question was observed in regions of proteins that interact with the immune system and that these regions would be expected to be conserved suggesting evolutionary pressure. Furthermore it was suggested that these new strains were not able to displace old strains in unvaccinated populations and that they were probably less fit suggesting that they had been selected for [104].

B. pertussis strains from all over the world share a great deal of similarity in genomic content, showing that the bacterium is largely monomorphic, although evolution continues, mostly though gene loss [105]. Gene content was speculated to be influenced by herd immunity provided by vaccination and in 2009 the first report of a strain of *B. pertussis* not expressing either Prn or PT was published [106]. The entire PT operon had been deleted, while the Prn gene had been disrupted by an insertion sequence (IS) element. Consequently the infant from whom the strain was isolated did not display leukocytosis, due to loss of PT, and the strain was found to be less pathogenic in a mouse model. Apart from these differences the strain was found to be genetically very similar to others currently circulating. It was suggested that the loss of PT in particular might pose problems for diagnosis since it is the presence of PT that discriminates from other *Bordetella spp*. Since this study isolates of *B. pertussis* not expressing Prn have been found all over the world including Finland [107], Japan [108], Australia [109] and the US [110]. The prevalence of Prn-deficient isolates was shown to be around 27% in Japan and 30% in Australia, while in strains collected after 2012 in the US 50% were Prn-deficient [108-110]. It has been shown that the odds of being colonised by a Prn-deficient strain of *B. pertussis* is 2 to 4-fold higher in vaccinated individuals demonstrating increased fitness of these isolates in these individuals, and there are thought to be no differences in clinical symptoms in disease caused by strains that express Prn and strains that don't [111]. Therefore the bacterium is thought to be evolving in response to the vaccine, by losing the ability to express a component of the acellular vaccine.

Following the 2012 outbreak in the UK, a large number of strains were analysed and it was found that the epidemic was polyclonal and caused by a number of distinct, closely related strains [112]. Furthermore, it was shown that genes coding for antigens that are included in the acellular vaccine are evolving at a higher rate than genes coding for other surface proteins. It was noted that this was happening even prior to the introduction of vaccines, which is to be expected since strains would be

under selection from the immune system anyway, but that this selection has increased since vaccination began. This is evidence that the acellular vaccine is having a direct influence on the pathogen, possibly contributing to vaccine evasion.

1.5 Metabolism of Bordetella pertussis

1.5.1 Early studies on metabolism of *B. pertussis*- requirements of carbon, nitrogen and sulphur

In the first detailed study of *B. pertussis* (then called *Haemophilus pertussis*) metabolism, washed suspensions were incubated with various carbon sources including carbohydrates, amino acids and fermentation products [113]. Catabolism was measured, indicated by either a rise in pH, oxygen consumption, carbon dioxide production or production of ammonium. Bacteria failed to oxidise carbohydrates at an appreciable rate and amino acids were the most rapidly oxidised. Specifically, serine, proline and aspartic acid were metabolised though results were variable. Of all the substrates tested glutamic acid was most rapidly utilised and it was concluded that this was probably the principle carbon source. This study was the first to define specific nutrient requirements of *B. pertussis* and until then, the bacteria were grown on a very general medium made up of casein hydrolysate, salts, starch, cysteine and nicotinic acid [114].

Later, the absolute requirements for *B. pertussis* growth were defined [115]. Nicotinic acid was needed for growth, and while the omission of any other factor did not abolish growth, removing purines, biotin and haemin reduced growth. *B. pertussis* could grow in very simplified conditions, with glutamic acid, proline or aspartic acid as sole carbon and nitrogen source, with cysteine as a source of sulphur (though this was dispensable in the presence of other sulphur containing compounds). Glutamic acid was the principle carbon source in the presence of other amino acids, but it could be replaced by either alanine, proline, aspartic acid or lactate although with a reduction in growth. Thus *B. pertussis* has very simple growth requirements, no one amino acid is essential for growth and the bacteria are able to grow using only one of three amino acids, though principally glutamic acid, as the sole carbon and nitrogen source, with nicotinic acid as the only vitamin added. It was suggested then that any

difficulties in growing the bacteria were probably due to inhibitory effects of other compounds in the media [115].

B. pertussis growth could be supported with cysteine, cystine or glutathione as the sole source of sulphur, though growth was significantly reduced with the latter [116]. This confirmed previous requirements and showed that growth of *B. pertussis* could be supported by only two amino acids (glutamic acid and cysteine) plus nicotinic acid.

1.5.2 Basis for currently used media

Stainer-Scholte medium is the basis for most media used today [117]. Good growth was obtained using a medium containing just Na-L-glutamate (10.7g/l), L-proline (0.24g/l), L-cystine (0.04g/), NaCl (2.5g/l), KH_2PO_4 (0.5g/l), KCl (0.2g/l), $MgCl_2 \cdot 6H_2O$ (0.1g/l), $CaCl_2$ (0.02g/l), $FeSO_4 \cdot 7H_2O$ (0.01g/l), Tris buffer (6.075g/l), ascorbic acid (0.02g/l), niacin (0.004g/l) and glutathione (0.1g/l). Furthermore, the cells grown in this medium were antigenic, and protective in a mouse model, though antigenicity declined over several passages of the cells in this medium showing that over time the bacteria become laboratory adapted. Since glutamic acid can be used as sole carbon source it can be inferred that *B. pertussis* is able to make all of the building blocks of the cell from this molecule. Cysteine is present only in small amounts as a source of sulphur.

1.5.3 Autoinhibitory free fatty acids

Early difficulty in growing *B. pertussis* could not be explained by its nutritional requirements as the bacteria are not fastidious and growth can be obtained in SS, described above with minimal nutrient requirements. Therefore there must be other reasons why good growth is difficult to obtain, such as presence of inhibitory molecules, either in the medium or produced during growth.

It had been known for some time that the reason why blood (or starch) were necessary components of early media was to absorb some toxic material, likely fatty acids and likely produced during growth [118]. It was then shown that even very low concentrations of fatty acids could inhibit growth, the most inhibitory being palmitic acid which was inhibitory at a concentration of 0.01mM [119].

It was shown that free palmitic, palmitoleic and stearic acids were found in the lipids extracted from cells of *B. pertussis* at cellular concentrations that were likely to be inhibitory [120]. These fatty acids were also found in the supernatant at significantly higher levels than uninoculated medium suggesting that they are released by cells during growth. It was speculated that these were the reason for several observations about the growth of *B. pertussis* including low growth yield and a marked deceleration in growth rate prior to stationary phase. Prior to this these observations couldn't be explained by limitation of nutrients or any other inhibitory products [121].

The benefits of adding heptakis ((2,6-*O*-dimethyl) β-cyclodextrin) to the medium were demonstrated [120]. This prolonged a higher growth rate, while not affecting the rate itself, and shortened lag phase, which was suggested to be due to contamination by free fatty acids. Also it was noted that at higher concentrations (more than 1g/l) heptakis might actually have adverse effects on growth, possibly by binding necessary nutrients, or because a small concentration of free fatty acids could be beneficial for growth. Since the production of autoinhibitory free fatty acids by bacteria is counter-intuitive, it was speculated that these fatty acids may have a role in pathogenesis, immune evasion or that they are a by-product of the production of other virulence factors [120].

1.5.4 Evidence for incomplete citric acid cycle

B. pertussis was grown on a variety of fermentation products with ammonium as a nitrogen source, and it was shown that only 2-oxoglutarate (α-ketoglutarate) could support growth [122]. Glutamate could be generated from 2-oxoglutarate, but apparently not from other citric acid cycle (TCA) products. *B. pertussis* must be able to synthesise fatty acids from glutamate (since it can grow with glutamate as sole carbon source), so it must be able to generate acetyl-CoA from oxaloacetate via pyruvate. It was noted that since growth ceased in the absence of glutamate or 2-oxoglutarate that *B. pertussis* could not form 2-oxoglutarate by condensing acetyl-CoA with oxaloacetate to form citrate, isocitrate and 2-oxoglutarate (see figure 1). The TCA cycle was therefore declared to be incomplete, though it was noted that this was in conflict with previous findings where it was shown that *B. pertussis* could

oxidise alanine, serine, proline and aspartate, which would require a complete TCA cycle [113].

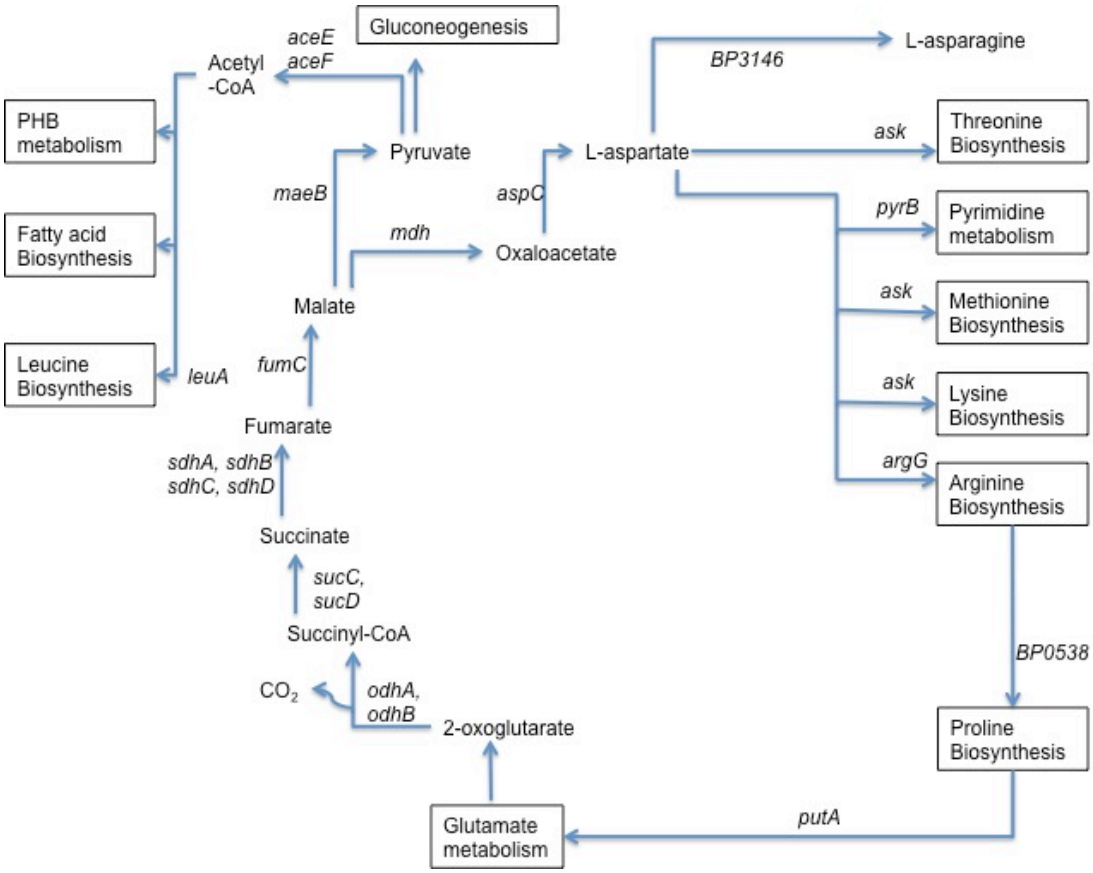


Figure 1- The theorised incomplete TCA cycle. 2-oxoglutarate was the only fermentation product that could support growth as sole carbon source. Acetyl-CoA needs to be produced from pyruvate, to feed fatty acid biosynthesis pathways, but *B. pertussis* was thought not to be able to form 2-oxoglutarate from oxaloacetate, done via citrate and isocitrate in a complete cycle.

Pyruvate, lactate, fumarate, succinate and 2-oxoglutarate were metabolised in the presence of glutamate (though pyruvate became inhibitory to growth at relatively low concentrations). It was noted that growth yields with 2-oxoglutarate, succinate or fumarate in combination with glutamate were lower than with glutamate plus lactate or glutamate only media. This lower biomass was accounted for by the finding that growth in the former three media led to the secretion of β-hydroxy-butyrate (β-HB) into the culture supernatant. This did not occur during growth in media containing glutamate plus lactate or glutamate only but did occur during growth in modified-SS (MSS) medium, which is SS medium supplemented with heptakis and casamino acids. β-HB appeared in the supernatant during late stages of growth and did not disappear, and its formation was used to explain lower biomass yields since β-HB is

not measured as part of the biomass and is a waste product. When substrates were present in concentrations lower than commonly used SS medium, β-HB was not detected. Thus, β-HB is a product of incomplete oxidation of substrates when they are present at higher concentrations. However, it wasn't clear why it appeared in the supernatant later in growth and not at the beginning, when substrate concentrations were highest. Globules containing poly-hydroxy-butyrate (PHB) were present in cells, which disappeared later on during growth coinciding with the appearance of β-HB in the medium. PHB was seen in cells grown on the lactate plus glutamate medium but β-HB was not detected, suggesting an alternative metabolic pathway for PHB in this medium. It was proposed that during log-phase growth at high substrate concentrations, more acetyl-CoA is generated than can be used, and CoA needs to be regenerated; so two molecules of acetyl-CoA are condensed to form acetoacetyl-CoA and CoA. β-HB is formed from the acetoacetyl-CoA and the second CoA is released on polymerisation of the β-HB monomer. PHB can be metabolised as substrates are depleted, the total amount probably determining how much is converted to biomass and how much is secreted as β-HB.

Thus, on medium with lactate in addition to glutamate, a higher biomass was achieved because β-HB was not secreted as waste. In addition, if enough lactate was present, net consumption of ammonium occurred, avoiding ammonium accumulation and pH rise due to the nitrogen:carbon imbalance that exists when metabolising glutamate only. Adding lactate along with glutamate redresses this imbalance since some of the glutamate molecules containing nitrogen are replaced with lactate, containing no nitrogen atoms.

1.5.5 Evidence of a complete TCA cycle

There is no functional glycolysis pathway in *B. pertussis* (genes for glucokinase and phosphofructokinase, as well as a part of the phosophotransferase system are absent) so *B. pertussis* is unable to metabolise sugars. The gluconeogenesis pathway is fully functional as is the non-oxidative branch of the pentose phosphate pathway, allowing *B. pertussis* to synthesise sugars for peptidoglycan, lipopolysaccharide and other glycans. In addition, growth can be supported by glutamate, proline or 2-oxoglutarate as sole carbon sources, suggesting that all amino acid biosynthesis pathways are functional, though growth is improved in complex media. It is necessary then for the

TCA cycle to be functional between 2-oxoglutarate and oxaloacetate to allow for synthesis of the aspartate family of amino acids. Pathways must also exist from this part of the TCA cycle to pyruvate to feed into gluconeogenesis, and fatty acid biosynthesis.

Despite evidence that an incomplete TCA cycle exists [122], orthologues of all genes for a fully functional TCA cycle are present, including genes for a type II citrate synthase, aconitase and isocitrate dehydrogenase thought to not function [1].

It was shown that not only are these genes expressed, but also that there are detectable enzyme activities, providing the first evidence for a fully functional TCA cycle in *B. pertussis* (see figure 2) [123]. It was also shown that an entirely functioning TCA cycle was in agreement with energy requirements for the growing bacteria. Curiously, the accumulation of PHB or free fatty acids reported previously [120, 122] were not seen, meaning that there was possibly some limitation in conditions under which these observations were reported which weren't factors in this study. A higher glutamate concentration was used in the medium as well as a different strain of *B. pertussis*.

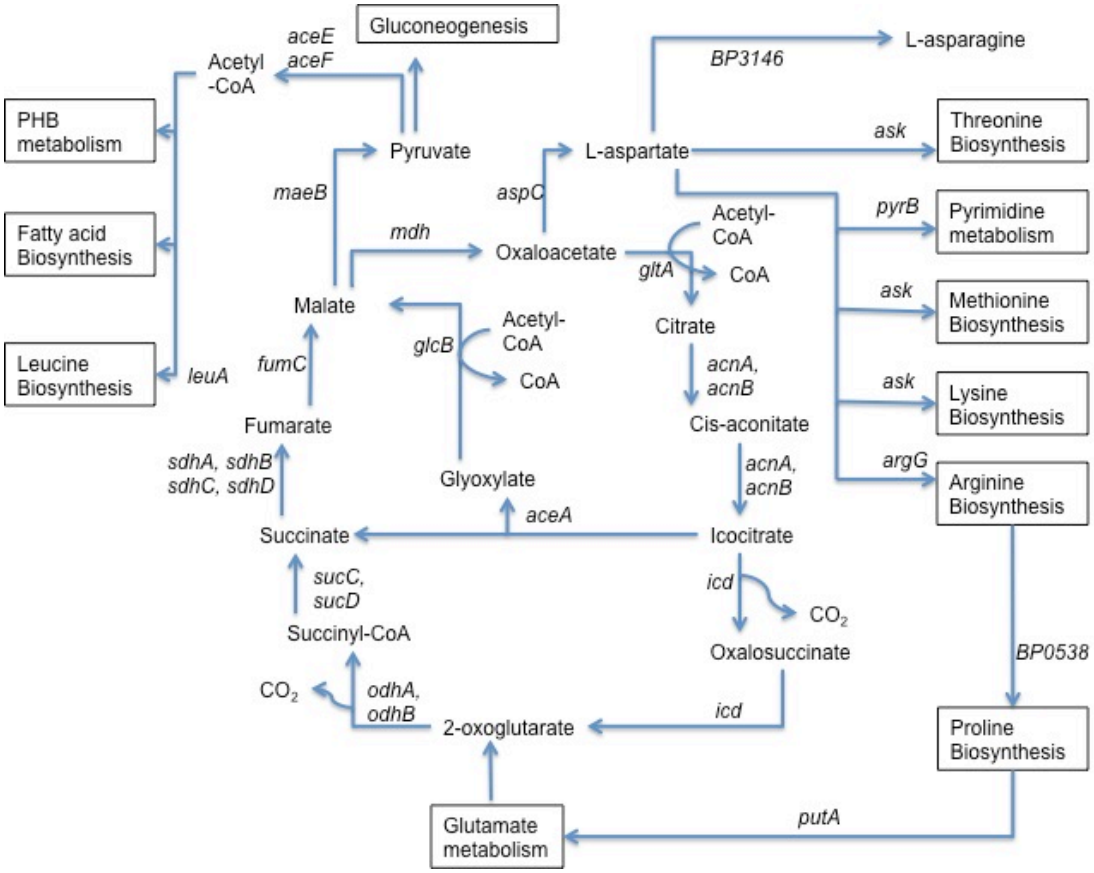


Figure 2- The complete TCA cycle of *B. pertussis.* Genes for citrate synthase (*gltA)*, aconitase (*acnAB*) and isocitrate dehydrogenase (*icd*) were found, and enzyme activities detected. Thus *B. pertussis* can form 2-oxoglutarate from oxaloacetate and has a complete TCA cycle.

The original proposal that *B. pertussis* has an incomplete TCA cycle came from the observation that the bacteria failed to metabolise citrate [122]. Since it is now clear that *B. pertussis* does have a fully functional TCA cycle (figure 2), the reason for lack of growth on citrate is not clear. It could be due to a reduced ability to import citrate, and while the import of radiolabelled citrate has been shown for *B. pertussis* [124], it remains to be shown whether or not enough can be imported to sustain growth. It is also important to mention that a different strain was used to propose an incomplete TCA cycle [122], though this strain has been shown through genomic hybridisation to include the three genes (citrate synthase, aconitase and isocitrate dehydrogenase) thought previously to be non-functional [105].

1.5.6 Fed-batch cultures- a context for vaccine production

The growth of *B. pertussis* has been studied in a fed-batch culture system, since this generally leads to higher cell densities, and more closely simulates culture conditions used in industry to produce vaccine antigens [125]. A low feed rate fed-batch was compared with a high feed rate fed-batch in which substrates should build up. It was shown that the final optical density in each case was 8.0 and 3.3 respectively (compared with 2.0 achieved in a batch culture), though the total amount of pertussis toxin (PT), a major component of the acellular vaccine, produced in the low feed rate culture was only 30% higher than in the high feed rate. This shows that PT, a major component of the acellular vaccine, production is not necessarily related to growth yield. The culture receiving the high feed rate had a lower biomass at the end of the feed phase, though no detectable waste products were found in the supernatant. Both cultures secreted free fatty acids in the supernatant (in agreement with [120]), though the maximum levels did not coincide with the end of the growth phase, suggesting they weren't the reason why the cultures stopped growing. To confirm this, cultures were grown with heptakis, a heptamer of glucose known to bind free fatty acids. The amount of free fatty acids, expressed as a percentage of total fatty acids, in the supernatant was the same in culture with heptakis as without, the final optical density was also the same. Therefore the secretion of toxic free fatty acids into the medium was not the reason why cultures stopped growing. The reason why *B. pertussis* could not maintain a higher growth rate in a fed-batch culture could be due to the accumulation of Na^+, due to the feed of Na glutamate. Furthermore, the production of PT stopped before growth did, suggesting a regulatory mechanism, which occurs more rapidly at higher growth rates.

In summary, the metabolism of *B. pertussis* is well defined. Media typically are glutamate-based with cysteine as a source of sulphur. Growth can be enhanced with addition of heptakis, which sequesters inhibitory fatty acids produced during growth, or mixtures of amino acids other than glutamate and some TCA cycle products can also be used. For purposes of vaccine culture, the production of antigens such as PT is not necessarily just linked to growth as there are regulatory mechanisms at play, which have a role in expression and secretion of virulence factors.

1.6 The Bvg Two-component System

1.6.1 Modulation

It was found that when sodium chloride was replaced by magnesium sulphate in Bordet-Gengou (BG) medium, colonies of *B. pertussis* appeared different from what had been observed previously [126]. A direct comparison between the two media revealed that colonies growing in the presence of magnesium sulphate appeared slightly larger and were not haemolytic compared to colonies grown on standard BG medium. These two states were named X mode (haemolytic, grown on standard medium) and C mode (non-haemolytic, grown on medium with magnesium sulphate). In addition, low temperature was found to switch cells to the C mode, and there was also described an I mode, which cells would inhabit within a narrow range of temperature and ion concentrations.

Crucially, it was found that the switch between X and C mode (and vice versa) occurred without mutation as it was reversible within 7-15 cell divisions. Thus the process of switching from X to C mode in the presence of magnesium sulphate or low temperature was termed modulation. In this C mode, cells did not agglutinate; have any surface antigen related to *B. bronchiseptica* or *B. parapertussis*. Furthermore, most antibodies obtained from recovered whooping cough sufferers were directed at the X mode suggesting that in the C mode *B. pertussis* was less virulent (or at least less antigenic).

1.6.2 Global regulation of virulence

The terms virulent and avirulent were used to describe the X and C modes respectively and it was shown that in addition to modulation, the avirulent phase could be attained by mutation. Some strains that were avirulent would remain avirulent even when grown on media that would normally induce the virulent phase [127].

Through construction of Tn5 mutants it was shown that virulence factors could be lost individually without affecting others, but two mutants were found containing single disruptions that affected expression of many virulence factors, inducing the avirulent phenotype [128]. A model was proposed by which there existed a trans-acting gene product that positively regulated expression of virulence factors.

When the bacteria were in the avirulent phase either due to environmental conditions or mutation, the gene product would not be made and expression of virulence factors would not be induced. In addition to this a reversible genetic event was described by which the bacteria could switch between the virulent and avirulent phases, which was postulated to be a form of phase variation. Questions remained about why the bacteria would have such a system that would affect virulence so globally, but it was suggested that it could be an extreme level of antigenic variation during infection or that the avirulent phase could allow the bacteria to survive in some as yet undiscovered environmental reservoir.

Later it was shown that regulation of virulence in *B. pertussis* was more complicated. Mutations in a gene, termed *vir*, did indeed explain phase variation of the bacteria between virulent and avirulent phases, but at least two trans-acting genes involved in this process were described. The second gene was termed *mod*, which was shown to be distinct from *vir* and characterised from strains of *B. pertussis* that constitutively expressed virulence factors even under modulating conditions such as the presence of magnesium sulphate or low temperature. The mutations in these strains were deemed to be in the *mod* gene. The *vir* and *mod* genes were speculated to be distinct from one another but to act cooperatively to either activate a set of genes (*vags*- virulence-associated genes) and repress another (*vrgs*- virulence repressed) [129].

1.6.3 Bvg two-component system

It was shown that if the *vir* locus from virulent strains was cloned and transferred into avirulent strains it caused them to express the virulent phenotype (smaller haemolytic colonies). Furthermore, if the cloned *vir* had come from an avirulent strain then an avirulent phenotype was maintained. This provided further evidence that *vir* was an activator of virulence and that avirulent mutants had a loss of function effect. The locus was sequenced and it was found that the difference between the virulent and avirulent spontaneous mutant strains was the presence of a seventh cytosine residue within a poly-C tract, causing a frame shift mutation. Importantly, it was noted that the sequence of the *vir* locus was significantly similar to a class of bacterial regulatory systems called two-component systems, one part being similar to the sensor component and the other being similar to the regulator. At this time little was known

about two-component systems but it was understood that the sensor part resided in the inner membrane and received a signal from the environment. The sensor then interacts with the regulator through phosphorylation, and the regulator then influences the expression of a set of genes. Thus the basis of both phenotypic modulation and antigenic variation had been discovered. The *vir* locus coded for a product that could act as a sensor to regulate the expression of genes on detecting a change in the environment- phenotypic modulation, but frameshift mutation in the poly-C tract could also lead to the same change in gene expression not dependent on environmental stimuli but a reversible mutational change- antigenic (phase) variation [130].

The full sequence of the *vir* locus was reported, and henceforth referred to as *bvg* (Bordetella virulence gene). The genes in this locus were designated *bvgA*, *bvgB* and *bvgC* and the products of these genes were required for expression of virulence factors. A different set of genes was repressed by *bvg*, and this repression was lifted upon phenotypic modulation, the functions of which were not known. The poly-C tract responsible for antigenic variation was found to be within *bvgC*, and insertional mutations in any of the three genes resulted in the Bvg- (avirulent) phenotype. It was again noted that the amino acid sequence suggested similarity to a two-component system but curiously that there were three genes within this locus. BvgA and BvgC were proposed to form the two-component system based on amino acid sequence but it was noted that BvgB did not contain either a transmitter or receiver domain for phosphorylation. A model was proposed whereby BvgB and the N-terminal domain of BvgC were localised to the periplasm, and it was suggested that they either interacted with each other or some common factor. The transmembrane region of BvgC spanned the membrane and the C-terminal region along with BvgA would be in the cytoplasm. BvgA was proposed to act as a transcriptional activator. Therefore BvgC would activate BvgA by phosphorylation. It was not clear how temperature would affect this system but it was proposed that magnesium sulphate could act to prevent phosphorylation of BvgA possibly through interaction with BvgB or BvgC [131].

The sequencing of the original *bvgB* and *bvgC* was found to have an error and these two genes were found to be one, coding for one sensor product. Thus the periplasmic sensor (formerly BvgB and BvgC) was renamed BvgS. It was also discovered in that study that the Bvg system was subject to positive autoregulation

(i.e. that it contributed to its own expression) [132]. The sequence TTTCCTA was found in the promoters for both *bvgA* and *fhab* (the virulence factor filamentous haemagglutinin), concluding that these sequences probably provided a binding site for a transcription factor and that this transcription factor was probably BvgA itself [133]. However, a global mechanism for Bvg-activation of virulence genes was still unclear since binding of BvgA to DNA upstream of *ptx* (coding for PT and known to be Bvg-regulated was not shown, and the sequence reported was not found in the *ptx* promoter.

The *mod* mutation previously described [129], thought to be distinct from *bvg* was later shown to be within *bvgS* [134]. Mutants in *bvg* were isolated that corresponded to single-nucleotide substitutions within *bvgS* that led to constitutive expression of virulence genes and insensitivity to magnesium sulphate, providing direct evidence that BvgS was indeed a sensor. All of the mutations mapped to a particular region of the protein, which was predicted to be located between the transmembrane region and the cytoplasmic signalling domain. This region was designated the linker. BvgS is made up of a perisplasmic region, three cytoplasmic regions and the linker. It is an unusual sensor kinase in that it contains transmitter and receiver domains. The constitutive mutations lock the protein into its active conformation making it insensitive to signals such as magnesium sulphate.

1.6.4 Mechanism of Bvg signal transduction

Later work pointed towards a model for Bvg signalling [135]. It was shown that the transmitter domain of BvgS could undergo autophosphorylation at the His^{729} residue *in vitro* using the ϒ-phosphate of ATP. This phosphate is passed onto the Asp^{1023} residue in the receiver domain before phosphorylating BvgA. BvgS is therefore an unusual form of sensor protein in that the transmitter and receiver domains are required for autophosphorylation to take place. The C-terminal region is also required. The model was later refined (shown in figure 3), confirming that BvgS does autophosphorylate at His^{729}, transferring the phosphate group to the Asp^{1023} in the receiver domain. A third phosphorylation event, the phosphorylation of His^{1172} in the C-terminal domain, was shown to be necessary for phosphorylation of BvgA [136].

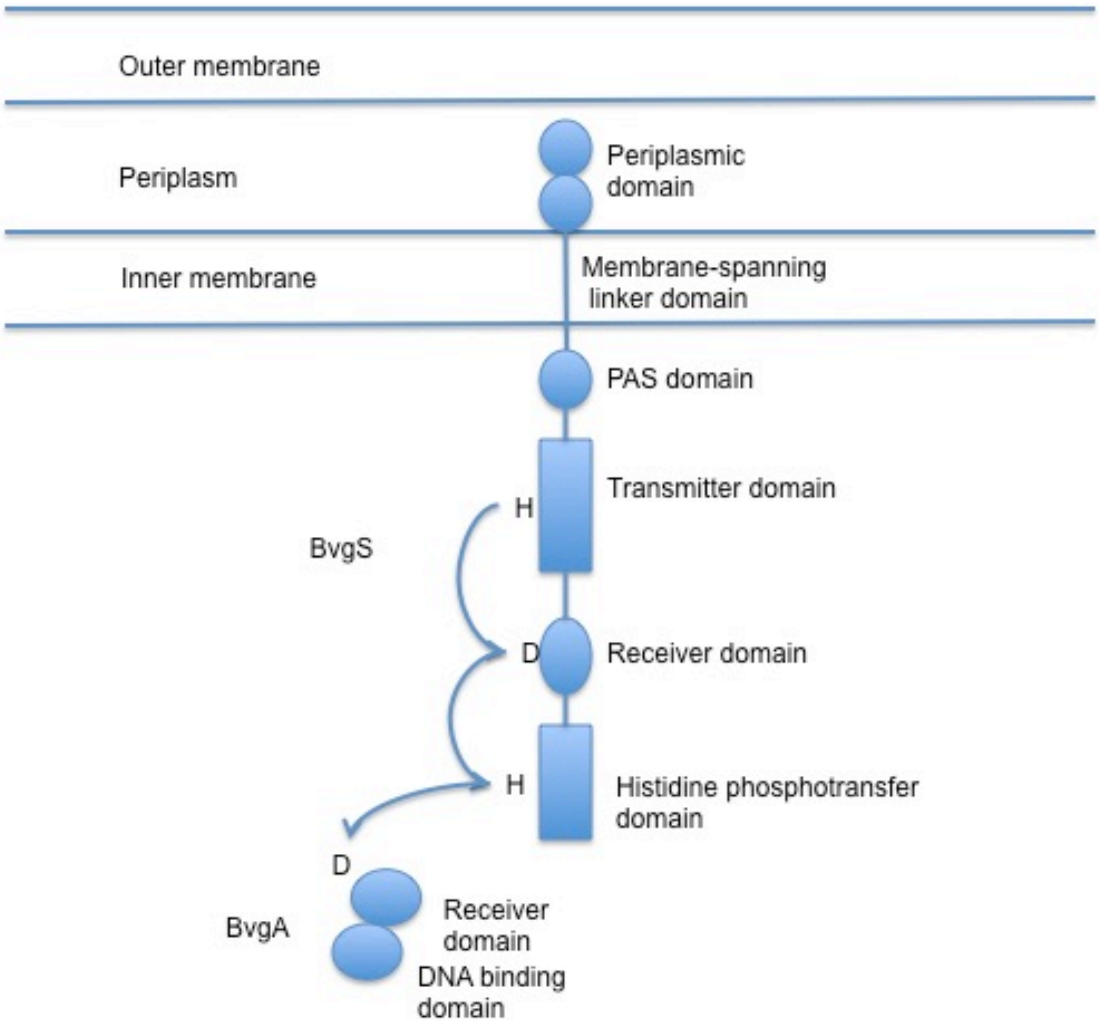


Figure 3- The Bvg system– BvgS contains regions that span the periplasm and inner membrane. The initial phosphorylation takes place on a histidine residue on the transmitter domain, and subsequently phosphorylates an aspartate residue in the receiver domain and a histidine residue in the phosphotransfer domain. From here BvgS phosphorylates an aspartate residue in the receiver domain of BvgA, which also contains a DNA-binding domain.

Both BvgS and BvgA were shown to have dimerisation properties. BvgS seemed to contain two regions with a propensity to homodimerise, the transmitter domain and the C-terminal domain, possibly explaining why the C-terminal domain was necessary for phosphorylating BvgA, unlike other sensor kinases. The complexity of the mechanism of signalling may reflect the ability to fine tune regulation of gene expression *in vivo* [137].

1.6.5 Gene regulation

It was still not yet clear whether all genes activated by Bvg were activated in the same way. Phosphorylated BvgA was shown to be sufficient to activate transcription from at least four Bvg-regulated promoters (*bvg*, *fha*, *cya* and *ptx*) [138]. This evidence moved away from the idea that accessory regulators might be needed in addition to BvgA in order to activate genes under regulation of Bvg. However, there did appear to be different levels of sensitivity of promoters to BvgA. Transcription was apparent from the *bvg* and *fha* promoters even with only unphosphorylated BvgA present, whereas transcription of *ptx* and *cya* was detectable only when phosphorylated BvgA was present [138].

It was shown how BvgA has different affinities for different promoters and that binding can be cooperative [139]. BvgA binds multiple times upstream of the *ptx* gene, starting at a higher affinity site further upstream, with more molecules binding progressively downstream. It was speculated that the numbers of binding sites and binding affinities would explain the difference between an "early" promoter such as *fha* and a "late" one such as *ptx*. The affinity of BvgA to a high-affinity site is increased on phosphorylation. BvgA dimerises and cooperative binding is induced with phosphorylated BvgA dimer binding every 21bp until RNA polymerase is recruited. Thus the early promoters are induced with a lower concentration of phosphorylated BvgA present, while late promoters have multiple BvgA binding sites and require cooperative binding and a greater concentration of BvgA in the cell to be turned on. This is an example of the complex nature of the Bvg system acting beyond a set of genes merely being on or off at one time or another.

1.6.6 BvgR

Another level of complexity in Bvg regulation was found. In addition to a number of genes (many of which code for virulence factors) that are maximally expressed when BvgAS is active there is a set of genes that are repressed in this phase [140]. A repressor protein, itself activated by BvgA, was expressed when BvgAS was active and bound *vrgs* in the coding sequences, blocking transcription. This protein is coded for by a gene lying adjacent to the *bvgAS*, which was named *bvgR*. A refined model was described in which BvgS autophosphorylates and in turn phosphorylates BvgA, BvgA acts as a transcription factor to activate transcription of a set of genes, many of which code for virulence factors but BvgA also upregulates transcription of *bvgR*,

coding for a protein which itself serves to repress a set of genes (*vrgs*), the functions of which are not known. Under modulating conditions BvgS does not autophosphorylate or phosphorylate BvgA, virulence factors are not transcribed, and neither is *bvgR*, meaning repression of *vrgs* is lifted. BvgR probably functions as a c-di-GMP phosphodiesterase so levels of this secondary messenger probably mediate repression of vrgs. RisA, a response regulator phosphorylated by RisK, mediates a second level of regulation of expression of vrgs. RisA could act directly or indirectly with levels of c-di-GMP to regulate expression of this gene set [141].

1.6.7 Bvgi

The potential for an intermediate Bvg phase (Bvgi) was explored [142]. The promoter of a gene, *bipA*, was described in which transcription was activated by cooperation of two phosphorylated BvgA dimers upstream of the RNA polymerase binding, and repressed by three more phosphorylated BvgA dimers within the coding sequence. In the Bvg- phase, BvgS is not active and the concentration of phosphorylated BvgA in the cell is too low to allow activation. Under Bvg+ conditions BvgS is active, the concentration of phosphorylated BvgA in the cell is maximal and the high and low affinity Bvg sites are occupied, resulting in activation and recruitment of RNA polymerase to the promoter site of *bipA* but also inhibiting transcription downstream of the recruitment site. When phosphorylated BvgA is at an intermediate level, in the Bvgi phase, only the high affinity sites upstream of the *bipA* promoter are occupied and transcription is initiated. Thus *bipA* was the first gene described to be maximally expressed in the Bvgi phase. The picture of the Bvg system functioning like an ON/OFF switch is an incomplete one; a clearer picture is one of a dimmer switch, allowing for more subtle changes in gene expression. *bipA* was expressed maximally at magnesium sulphate concentrations of between 20 and 35mM and undetectable when magnesium sulphate concentrations were above 40mM or below 15mM. It was hypothesised that BipA may be needed very early on in establishing infection, before adhesins (*fha*, an “early” Bvg promoter) and then toxins (*ptx*, a “late” Bvg promoter). BipA may be disadvantageous later in infection since its expression is repressed. The subtle gene expression changes allowed for by the Bvg system may reflect the temporal and spatial niches during infection (early colonisation vs. late infection;

colonisation in the lower temperature higher temperature nasal cavity vs. the trachea, or in other niches not known).

1.6.8 The Bvg regulon- species variation

The idea that the Bvg system fine-tunes expression to suit a niche, temporal or spatial was agreed with, and it was shown through expression studies that the Bvg+ phase was highly conserved between *B. pertussis* and *B. bronchiseptica* [143]. Gene expression profiles in the Bvg- phase, however, were quite different between the two species. In *B. bronchiseptica* the Bvg- phase appears to be important for transport of substrates, motility and chemotaxis, suggesting a nutrient scavenging role for this phase in the environment. The Bvg- phase of *B. pertussis* appeared to have a less varied role, reflecting host restriction of this species and limited ability to survive outside of the host. It was shown that a random loss of Bvg- phase genes had occurred in *B. pertussis* compared to *B. bronchiseptica* suggesting that on host restriction *B. pertussis* has less of a need for a scavenging Bvg- phase and that this phase may well be an evolutionary remnant in *B. pertussis*. The Bvgi phase was shown to be quite different between the two species. It was speculated therefore that this phase represents species-specific functions, suggested to be survival in a microaerophilic condition for *B. bronchiseptica* due to the expression of a number of genes coding for terminal respiratory oxidases in this phase. In contrast, a role for the Bvgi phase in *B. pertussis* was not clear and it was suggested that this too might be an evolutionary remnant in this species.

In addition to the classical virulence genes, eight autotransporter genes were classified as Bvg-activated as well as several encoding iron acquisition proteins and a potential novel toxin. Of the fimbrial genes, *fim2* and *fimA* were activated in the Bvg+ phase in *B. pertussis* and *B. bronchiseptica* as well as *fim3*, *fimX* and *fimN* in *B. bronchiseptica*. As expected, genes for the production and secretion of pertussis toxin were strongly Bvg-activated in *B. pertussis*. Other Bvg-activated genes in this species included a porin (*BP0267*) and an iron-transporter (*bfrE*).

Only 13 genes were found to be Bvg-repressed across both species. This included the *wlb* genes for synthesis of the band A trisaccharide of LPS, suggesting preferential production of band A LPS in the Bvg- phase. In addition to this, in *B. bronchiseptica* Bvg-repressed genes included those encoding two autotransporters,

two iron-acquisition proteins, and the chemotaxis and flagellar machinery. In *B. pertussis* only, five genes in the putative capsule biosynthesis locus were found to be Bvg-repressed, suggesting a role in the Bvg- phase. Only a few genes were found to be regulated in a similar fashion to *bipA*, that is, maximally expressed in the Bvgi phase. The Bvg regulon was shown to be flexible within species with 20% of Bvg-repressed genes from Tohama I (a lab strain of *B. pertussis*) shown to be also Bvg-repressed in a recent clinical strain. In addition 60% of the Bvg-activated genes were also Bvg-activated in the clinical strain showing a greater conservation of these genes within *B. pertussis* species. The reason for intra-species variation was unclear but could be due to continual evolution of *B. pertussis* driving genes out of Bvg regulation by promoter mutation or variation in further transcription factors, themselves regulated by Bvg.

1.6.9 Bvg-

Still not much is known about the Bvg- phase of *B. pertussis*, either in terms of genes expressed maximally in this phase or what its role is during infection. *B. pertussis* is a human restricted pathogen, and since virulence factors, including mechanisms of immune evasion, are not expressed in the Bvg- phase it is unclear what the Bvg-phase of *B. pertussis* would be used for, or indeed if it is used, there is evidence that this phase may be an evolutionary remnant.

1.7 Aims of this Study

Despite the existence of a vaccine and the sharp decline in incidence during the second half of the 20th century, the resurgence of disease makes studying *B. pertussis* vital. Production of purified antigens for use in the acellular vaccine requires large cultures, which take a long time to reach and risk the build-up of mutant "non-producers" that do not express the vaccines antigens [144]. Much is known about the metabolism of *B. pertussis* but little is understood about the phenotypic behaviour of the Bvg- phase beyond casual observation. This study aims to characterise the growth of BP536, a derivative of vaccine strain Tohama I, in the Bvg- phase, including analysis of key metabolites in order to observe how growth is effected by activity of the Bvg system.

The advent of genomics has revolutionised the ability to study evolution and epidemiology of *B. pertussis* [145] but there exists a bottleneck between discovery of new genes and assigning functions. This study aims to use state of the art genomics-based approaches such as TraDIS and RNAseq to inform about physiological differences between Bvg+ and Bvg- phase *B. pertussis* that can then be validated using more traditional techniques.

The ultimate aim is to evaluate whether or not there is a rationale for using information about Bvg- phase growth to improve the growth of antigen-producing vaccine cultures, which would reach an acceptable yield of culture faster and avoid build-up of "non-producers".

Chapter 2- Materials and Methods

2.1 Strains and culture conditions

B. pertussis was cultured on charcoal agar (Oxoid, Basingstoke, UK) for 72 hours at 37°C. Liquid cultures were grown in Stainer-Scholte medium (Stainer, 1970) supplemented with 1g/l (2,6-di-O-methyl)-β-cyclodextrin (heptakis) (Sigma, Gillingham, UK) when stated. *B. pertussis* BP536 is a one-step streptomycin resistant derivative of Tohama I [1]. Magnesium sulphate added to the medium to a final concentration of 50mM was used to modulate to the Bvg- phase.

E. coli was cultured for 18 hours on LB (Lennox) agar or broth (Sigma, Gillingham, UK) at 37°C. Vectors were transformed into 5-α high efficiency competent *E. coli* (New England Biolabs), *E. coli* strain ST18 was used as conjugation donor strain [146].

Antibiotic	**Concentration used**
Kanamycin	50μg/ml
Gentamycin	5μg/ml (*E. coli*), 30μg/ml (*B. pertussis*)
Spectinomycin	100μg/ml

Table 1- Antibiotics and concentrations used.

2.2 Growth assay

BP536 was resuspended in 30ml SS broth in a 200ml Erlenmeyer flask and incubated for 24 hours at 37°C with shaking. After this time the bacteria were pelleted by centrifugation at 4500xg, washed with PBS, pelleted and resuspended in PBS to an optical density at 600nm (OD_{600}) of 0.5. 50μl of this resuspension was added to 200μl SS broth in wells of a clear, sterile, round-bottom 96-well plate (Corning) meaning that cultures in the plate were started at an OD_{600} of 0.1. Bvg+ conditions were without magnesium sulphate, while Bvg- conditions were with 50mM magnesium sulphate. All of the outside wells of the plate contained 200μl water to minimise the risk of evaporation of the cultures. The plate was incubated at 37°C in a plate reader (FluroStar Omega, BMG Labtech) with shaking reading absorbance at 600nm every 15 minutes for up to 70 hours.

2.3 CFU viable counts

10µl of three of the Bvg+ cultures and three of the Bvg- cultures was removed and diluted in 990µl of PBS. 10-fold serial dilutions were performed and 100µl of three of the dilutions (10^5, 10^6 and 10^7 fold dilutions) was spread onto charcoal agar. The plates were incubated at 37°C for 5 days after which the colonies on the plates containing countable numbers of colonies were counted and the CFU/ml of the cultures calculated. This procedure was performed at the start of the growth assay and repeated a further five times during the assay. The same six wells were assessed for viable cell count each time.

2.4 Biomass measurements and glutamate consumption

BP536 was resuspended from a plate in 100ml of SS broth in 250ml Erlenmeyer flasks and incubated at 37°C for around 40 hours. Bvg+ and Bvg- cultures were grown. At intervals 15ml of culture was removed from the flask and centrifuged at 4000xg to pellet the cells. The supernatant was removed, filtered and stored at -80°C. The cell pellets were freeze-dried to obtain a dry pellet, which was weighed to determine dry cell weight. The supernatant samples were assayed for glutamate concentrations using the glutamate assay kit (fluorometric) (Abcam, Cambridge, UK; ab138883). Glutamate consumption in millimoles per gram during exponential phase was calculated by dividing the drop in glutamate concentration by the increase in biomass.

2.5 Metabolite assays

Five times during a growth assay four of the Bvg+ cultures and four of the Bvg- cultures were removed from the plate, replaced with 250µl water, and centrifuged at 16000xg for 10 minutes. The supernatant was removed and frozen at -80°C.

The supernatants were assayed for concentrations of glutamate, ammonium, β-HB and free fatty acids using kits provided by Abcam, Cambridge, UK (ab83360, ab83389, ab83390 and ab65341 respectively) using the protocols provided.

2.6 Primers and vectors

Primer	Sequence (BsaI site in bold)	Product
mreB_KRight_F	AAAA**GGTCTC**TCGAGAttcgggggcgttgg	*mreB* flanking right region
mreB_KRight_R	AAAA**GGTCTC**CATGTGcatgggagctcagctagatt c	*mreB* flanking right region
mreB_KLeft_F	AAAA**GGTCTC**AGGTCtgagcctgtctcgcg	*mreB* flanking left region
mreB_KLeft_R	AAAA**GGTCTC**GAACTgggcggctcgtacagc	*mreB* flanking left region
GG_acat_Kan_F	AAAAAA**GGTCTC**CACATgacgtcttgtgtctcaaaat ctc	Kanamycin cassette
GG_Kan_ggtc_ R	AAAAAA**GGTCTC**AGACCttagaaaaattcatccagc atc	Kanamycin cassette
petA_LF	AAAA**GGTCTC**TCGAGAgcagcacggtgacatcttcc	*petABC* left flanking region
petA_LR	AAAA**GGTCTC**GATGTGcatcttcctatccttgttgatgt gtcc	*petABC* left flanking region
petA_RF	AAAA**GGTCTC**TACATAtgacgcccgcgtcg	*petA* right flanking region
petA_RR	AAAA**GGTCTC**GAACTAagtacgacatctggcccca	*petA* right flanking region
petC_RF	AAAA**GGTCTC**TACATAtaacccgcattgcccc	*petABC* right

		flanking region
petC_RR	AAAA**GGTCTC**GAACTAtgctctggcgggcct	*petABC* right flanking region
Grp_LF	AAAA**GGTCTC**TCGAGActcgggcacgtcgc	*grp* right flanking region
Grp_LR	AAAA**GGTCTC**GATGTGtagaggggggcgccg	*grp* right flanking region
Grp_RF	AAAA**GGTCTC**TACATA**caa**atagcgtcaggtgtagcgg	*grp* left flanking region
Grp_RR	AAAA**GGTCTC**GAACTAgcagcacacctttcctgctgc	*grp* left flanking region

Table 2- Primers used in this study. These were primers used to amplify flanking regions of genes to make knockout mutants. BsaI sites in the primers are shown in bold.

Vector	**Selection**	**Source**
pCR8GW:GG	Spectinomycin	Made in house
pSS4940GW	Gentomycin	Made in house

Table 3- Vectors used in this study. pCR8GW:GG, adapted from pCR8GW (Invitrogen, California, USA). Vector pSS4940 was a gift from Scott Stibitz and is nonreplicative in *B. pertussis*. It contains a gene for synthesis of the I-*Sce*I restriction enzyme under the control of the *ptx* promoter and a gentamycin resistance gene. The GW cassette was ligated into the MCS using the Gateway vector conversion system (Invitrogen) and contains a chloramphenicol resistance gene and the *ccdB* gene, which targets gyrase and is lethal when expressed.

2.7 PCR

PCR was performed as a 50µl reaction with the following components; 1x Q5 reaction buffer, 0.2mM dNTPs, and 0.5µM forward and reverse primer. 0.5µl of the reaction was Q5 high-fidelity polymerase and 10µl was high-GC enhancer (both NEB, Hitchin, UK). Template DNA was made by touching a tip to cells grown on an agar plate and then dipping into 200µl DNAse-free water, before boiling at 100°C for 10 minutes and centrifuging at 16000xg for 10 minutes. 100µl of the liquid was taken and stored at -20°C. 1µl of this template was used in a 50µl reaction.

2.8 Golden gate reaction

100ng of vector pCR8GW:GG was mixed with equimolar amounts of each purified fragment and the golden gate reaction was performed [147]. Briefly, the DNA was incubated for 25 cycles of 3 minutes at 37°C and 4 minutes at 16°C followed by one cycle of 5 minutes at 50°C and 5 minutes at 80°C, with 1mM ATP, 20 units of BsaI-HF, 2000 units of T4 high concentration ligase and 1x cutsmart buffer (all NEB).

2.9 Gateway reaction

The pCR8 vectors following completion of the golden gate reaction were mixed with pSS4940GW in a Gateway LR Clonase II (Invitrogen Cat. No. 11791-020) reaction as per the recommended protocol and incubated at 25°C for 18 hours to generate pSS4940 vectors containing an insert.

2.10 Conjugations and selections for recombination events

pSS4940 vectors, containing inserts for making knockout mutants, were transformed into *E. coli* strain ST18 for conjugation into *B. pertussis* strain BP536 as described by Thoma [146]. BP536 and ST18 were grown on plates. One third to one half of the growth from a plate of BP536 was swabbed with a 2mm blob of donor ST18 strain containing pSS4940 onto charcoal agar containing 50mM $MgSO_4$ and 10mM $MgCl_2$. This plate was incubated at 37°C for 3-4 hours after which the cells were swabbed

onto selective charcoal agar containing 50mM $MgSO_4$ and incubated at 37°C for 4-5 days until single colonies appeared.

Selection with gentamycin selects for integration of the plasmid onto the chromosome of BP536, since it is nonreplicative in *B. pertussis.* The $MgSO_4$ gives growth in the Bvg- phase. In this condition the *ptx* promoter in front of the I-*Sce*I gene is not activated. A single recombination event is therefore selected for during which the plasmid has integrated into the chromosome and the cell contains both the mutant and the wild-type allele. These are single crossovers. Colonies were typically passaged twice until enough cells were obtained to freeze at -80°C in 20% glycerol.

One frozen clone was chosen and grown on charcoal agar. Without $MgSO_4$ the promoter in front of the I-*Sce*I gene is activated, which is a lethal event. Thus, growing clones without $MgSO_4$ selects for clones that don't contain the integrated plasmid and have resolved. This is the second recombination event, during which either the wild-type allele is looped out, or reversion to the wild-type genotype takes place.

2.11 Viability counts of the *mreB* mutant

To test viability under Bvg- phase conditions the *mreB* and BP536 were grown on charcoal agar for 4 days and resuspended to an OD_{600} of 1.0. Serial dilutions were plated on charcoal agar in Bvg+ and Bvg- conditions. The numbers of colonies after 7 days of growth were counted.

2.12 Preliminary growth of the *mreB* mutant

Two different clones of the *mreB* mutant were grown on plates alongside BP536. Cells were resuspended in 15ml SS broth with and without heptakis at 1g/l to an OD_{600} of 0.2 in 50ml sterile centrifuge tubes. Growth was measured periodically (twice a day) using a spectrometer to measure absorbance at 600nm.

2.13 Measurement of MIC of ampicillin

BP536 was plated on charcoal agar to give a lawn in Bvg+ and Bvg- conditions and an E-test strip for ampicillin was placed on the agar with sterile tweezers. The plates

were incubated at 37°C for 4 days and the point at which growth was inhibited was read off the strip.

2.14 Transmission electron microscopy

Strains were grown in SS broth in 50ml conical tubes until exponential phase was reached (1-2 days), after which cells were pelleted at 4500xg for 10 minutes and resuspended in PBS. Cells were washed in 0.1M sodium cacodylate buffer (pH7.3) and resuspended in the fixative solution (0.1M sodium cacodylate, 2.5% glutaraldehyde, 2% paraformaldehyde, 2.5mM calcium chloride) for 2 hours at room temperature and overnight at 4°C.

The next day the fixative was removed and cells were washed 3 times in 0.1M sodium cacodylate. Following the final wash, the wash solution was removed and replaced with 1% aqueous osmium tetroxide with 1% potassium ferrocyanide for 1 hour at room temperature. The cells were then washed three times with distilled water, before being stained with 2% aqueous uranyl acetate for 1 hour in the dark.

The cells were dehydrated in increasing concentrations of acetone (30, 50, 70, 90 and 95%, twice each) and finally in 100% dry acetone. Following this the cells were infiltrated with Spurrs epoxy resin, placed in moulds and polymerised in an oven at 70°C for 8 hours. Samples were then sectioned and imaged by TEM (Model JEOL JEM1200EXII, Tokyo, Japan) operating at 80kV. Sections were approximately 100nm in thickness. Multiple images were taken and all cells across different images were measured to get a representative sample.

Images of cells were measured using ImageJ. Lengths of cells were taken to be the largest distance from one side of the cell to the other; the width was taken to be perpendicular to this point. Volume of cells was also measured, with the volume taken to be similar to that of a cylinder, using the equation $V=\pi r^2 h$ where V is volume, r is radius (half of the width), and h is height (length).

2.15 Proton motive force (PMF) assay

The BacLight bacterial membrane potential kit (Thermo) was used to provide a relative measure of membrane potential by flow cytometry. Flask cultures of BP536 were grown in both Bvg+ and Bvg- phases, after 24h of growth cultures were diluted

in 1ml of filtered PBS to achieve of OD_{600} 0.01. For each culture an untreated sample was used to locate cells, a treated sample was obtained by adding 10µl of 3mM carbocyanine dye 3,3'-diethyloxacarbocyanine iodide ($DiOC_2(3)$), and a dissipated PMF control sample was obtained by adding 10µl of 500µM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) followed by 10µl of 3mM carbocyanine dye ($DiOC_2(3)$). The samples were analysed by flow cytometry recording forward scatter, side scatter, and fluorescence using filters for PI and FITC. Ten thousand events were recorded for each measurement and the ratio of red/green fluorescence was measured. $DiOC_2(3)$ exhibits green fluorescence but shifts to the red end of the spectrum as the dye molecules self-associate at higher cytosolic concentrations caused by large membrane potentials. The dye is taken up into the cell in a manner that is dependent on the proton motive force. Thus the ratio of red/green fluorescence is proportional to the proton motive force (PMF). The sample dissipated with CCCP is measured as a control to show background fluorescence that is not due to PMF.

The BacLight membrane potential kit was also used to measure PMF of the *petABC* mutant vs. BP536 grown on charcoal agar in Bvg+ and Bvg- conditions. Strains were grown for 3 days at 37°C, passaged and grown again for 2 days at 37°C, after which cells were resuspended in filtered PBS and to an OD_{600} 0.01 and assayed as described above.

2.16 TraDIS

BP536 was conjugated with ST18 containing plasmid ep1 as described above and selected on charcoal agar with 50mg/ml kanamycin and incubated for 5 days at 37^{o}C. Nine independent conjugtions were performed and each plated on multiple 130mm diameter agar plates. This allows construction of a high-density mutant library. Colonies were recovered in PBS and genomic DNA was extracted to give pooled genomic DNA for Bvg+ and Bvg- phase conjugations. Conjugations were performed by Jerry King.

2.17 Sequencing of transposon mutant libraries

Following preparation of genomic DNA, DNA was 2µg fragmented by Covaris to an average size of ~300bp. Fragments had adapters ligated (IDT, Leuven, Belgium).

PCR enrichment of transposon-containing fragments was performed with primers homologous to the ends of the transposon and an adapter-specific primer. PCR products were pooled and sequenced on a HiSeq2500. Sequencing and analysis to determine insertion indices, identify essential, ambiguous and fitness affected genes was done by the Sanger Institute.

2.18 Cultures for RNA isolation

BP536 was resuspended in 15ml of SS to an OD of 0.15. 0.6mM niacin was added to induce the Bvgi phase, and 50mM $MgSO_4$ to induce the Bvg- phase. Four cultures each of Bvg+, Bvgi and Bvg- phase BP536 were incubated at 37°C until they reached an OD_{600} of between 0.5 and 1.0 (taken to be exponential phase).

2.19 Isolation of RNA

An amount of cells equivalent to 1ml of an OD_{600} 4.0 was pelleted and resuspended in 1.5ml Tri reagent. RNA extraction was then performed using Direct-Zol RNA Miniprep Kit (Zymol Research) according to instructions, enriching for small-RNAs, as described in the protocol, and with two elution steps of 35µl each.

2.20 DNAse treatment

DNAse treatment was performed using Turbo DNA-free kit (Ambion). The rigorous treatment was performed according to the protocol using 2µl of enzyme and incubating at 37°C for 1.5hrs, before another 1µl enzyme was added and the samples incubated for another 2hrs. RNA concentration was measured for each sample using a Qubit Fluorometer and the yields of RNA calculated. All yields were between 8.5 and 14.5µg. RNA was frozen at -80°C until needed.

2.21 Validation of RNA for RNAseq

1µg of RNA was used in a first strand synthesis reaction using the Protoscript taq RT-PCR Kit (NEB). Random primers were used to synthesise cDNA for each RNA sample as instructed. Quantitative-PCR (qPCR) was used to measure the relative

amounts of transcripts in each sample relative to standard housekeeping genes. Primers used are shown in table 4.

Primer	Sequence
adk Forward	GCTACCTGTTCGACGGTTTC
adk Reverse	TGAAGCGTACGTGGTAGCTG
tyrB Forward	TGTTCATCAGCTCGTCGTTC
tyrB Reverse	GCCAGTTCATTTTCCCAGAG
ptxA Forward	GACCACGACCACGGAGTATT
ptxA Reverse	CGCGATGCTTTCGTAGTACA
fhaB Forward	TCTCGCACAACAAGTTCCAG
fhaB Reverse	CCTTGCCATAGACTTCGAGC

Table 4- Primers for qPCR

Standard curves for each primer pair were generated using 10-fold dilutions of genomic DNA from BP536 prepped with the GenElute Bacterial Genomic DNA Kit (Sigma). Each reaction consisted of 400nm of each primer, 1x SYBR Green Universal Master Mix (Applied Biosystems) and water to a final volume of 20µl, to which 5µl of template was added (genomic DNA or cDNA). Reactions were performed on a StepOnePlus Real-Time PCR System (Applied Biosystems). Conditions were an initial 95°C for 10 mins followed by 40 cycles of 95°C for 15s and 60°C for 1 min. Melt curve analysis was done following this with an additional 95°C for 15s and 60°C for 1 min.

2.22 RNAseq

Sequencing of RNA, mapping and analysis of data including fold-change of each gene between Bvg+ and Bvg- phase was performed by Eurofins (Ebersberg, Germany).

2.23 Analysis of the growth and glutamate consumption of the *grp* mutant

The *grp* mutant and BP536 were grown in a 96 well plate as described above. There were 15 cultures of BP536 and the mutant in both the Bvg+ and Bvg- phases. During the growth assay supernatant from three cultures for each condition was extracted periodically and stored as described above (see Metabolite assays). This was done a total of five times throughout the assay. Medium from the initial cell suspension used to inoculate medium inside the wells was harvested too, to measure the amount of glutamate in the medium before growth had commenced.

Chapter 3- Metabolism of *Bordetella pertussis* is dependent on Bvg phase

The growth of *B. pertussis* has been well characterised, including finding that the bacteria have simple nutrient requirements [115-117]. Difficulty in growing *B. pertussis* has been attributed to the build up of inhibitory compounds during growth such as fatty acids [118, 120] and ammonium [122]. Most of the metabolic studies of *B. pertussis* have been done in, *B. pertussis* in the Bvg+ phase, since it is this phase in which the bacteria grow *in vitro* when grown at 37°C and modulatory compounds are not added. It is the Bvg+ phase that produces virulence factors so it is in this phase that growth of *B. pertussis* is studied in the context of vaccine production, and work has been done with the aim of improving yield of antigens such as *ptx* during growth [121, 125, 148, 149].

The metabolic capabilities of the Bvg- phase, however, are unstudied, though it is known that cells that have a spontaneous mutation in *bvgS* accumulate during growth of cultures due to greater fitness of these mutants [144]. This suggests that there is something different about the way that *B. pertussis* grows that is dependent on Bvg phase. It could be that in the Bvg- phase the cells are fitter because they do not have the burden of producing virulence factors. However, the Bvg regulon is large and there are a number of genes that are maximally expressed in the Bvg- phase [143], so it is possible that there is a different type of growth in this phase. The aim of this study was to investigate the difference in growth between Bvg+ and Bvg- phase *B. pertussis*, testing for differences in metabolite production, and to investigate why it is that Bvg- phase *B. pertussis* has a fitness advantage over cells in the Bvg+ phase in vaccine cultures.

3.1 Results

3.1.1 Growth curves of *B. pertussis* in Bvg+ and Bvg- phases

Growth of BP536 in the Bvg+ and Bvg- phases is shown in figure 4. Data shown is the average of 27 cultures at the start of the assay, diminishing by 4 each time cultures were sacrificed for measurements of metabolites, leaving 11 cultures of each at the end of the assay.

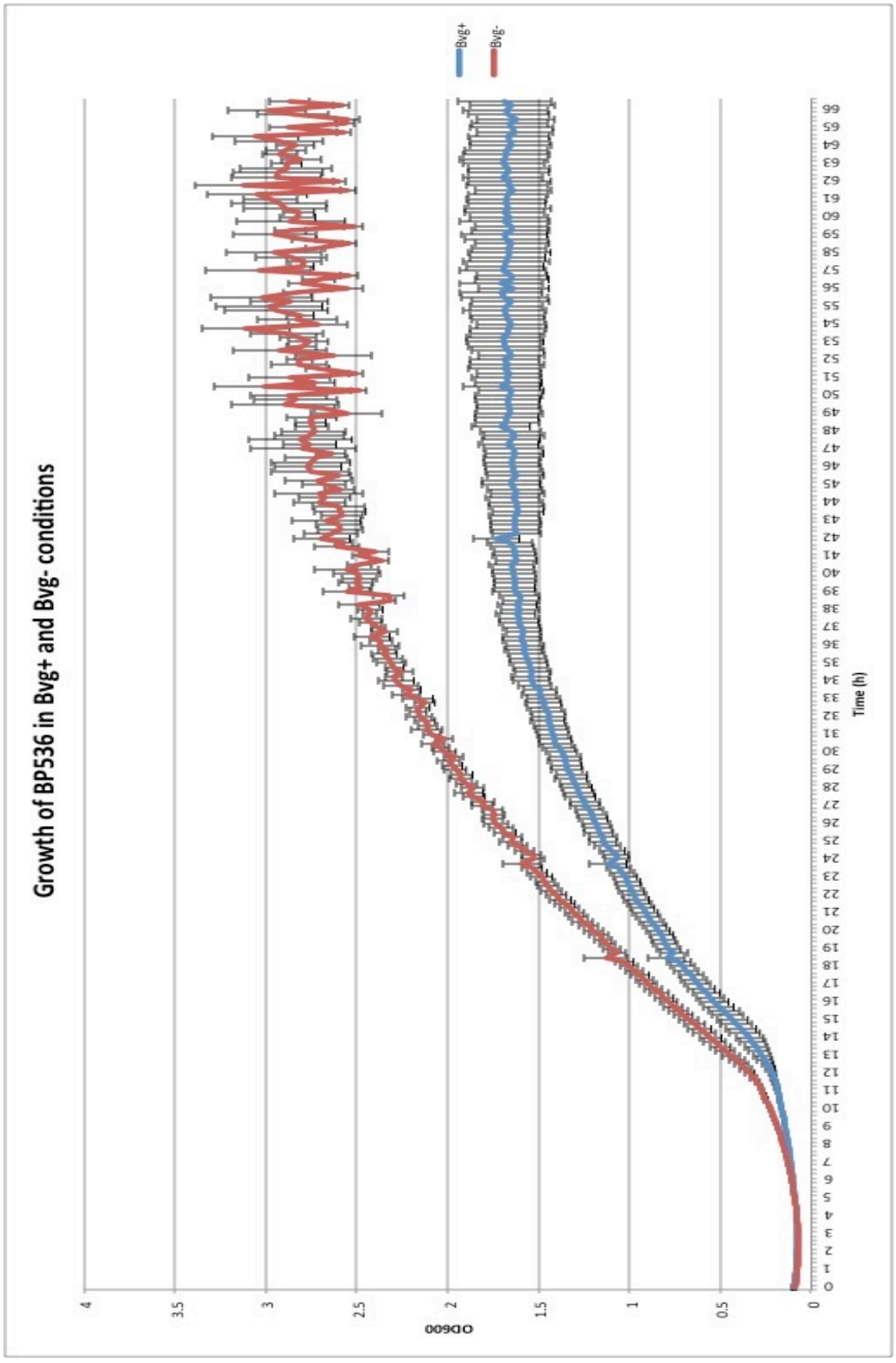


Figure 4- Growth curve of BP536 grown in SS broth in the Bvg+ and Bvg- phases. BP536 was grown in a 96-well plate until stationary phase was reached. Features of growth displayed include a relatively long lag phase, a progressive decrease in growth rate throughout log phase and a poor final yield which is improved in the Bvg- phase. Error shown is standard deviation

Growth of *B. pertussis* is characterised by a long lag phase and short period of exponential growth. A low final yield is also a problem for vaccine cultures.

This growth assay is not representative of cultures grown for vaccine production, which are grown in batch culture in fermenters on a much greater scale. The medium used for vaccine production is typically modified-SS (MSS), which contains heptakis and casamino acids. The purpose of this experiment was to use a chemically defined medium to compare Bvg+ and Bvg- growth in the simplest way possible. Figure 4 shows a clear difference between Bvg+ and Bvg- phase growth, which will be discussed.

3.1.2 CFU counts

Optical density is only an indirect measurement of bacterial growth, correlating turbidity with cell division. To check that optical density was a valid way of measuring bacterial growth of *B. pertussis* CFU counts were carried out. 10µl of culture was removed from three Bvg+ and three Bvg- cultures at six time-points during growth, including at the beginning of the assay.

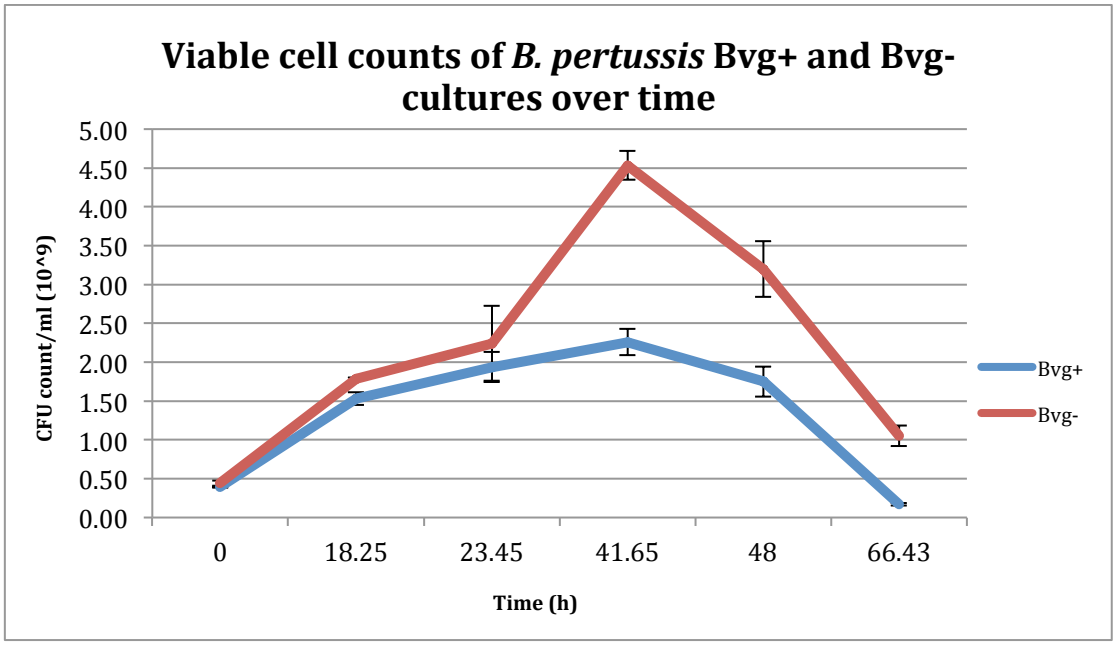


Figure 5- Viable cell counts of BP536 in Bvg+ and Bvg- phase. Samples were taken at six time points during the growth curve and plated for viable cell counts. The data is an average of three biological replicates (three separate cultures from within the same 96-well plate), the same cultures were used to measure CFU at different time points. Error is standard error of the mean.

The time points at which CFUs were measured correspond to early, mid and late stage exponential phase growth, and early and late stationary phase. The growth as measured by CFU count corresponds well with OD, at least for the first half of growth. Figure 5 shows that growth reaches the maximum CFU count at between 41.65 and 48 hours, and the maximum yield for Bvg- growth was 4.53×10^9 CFU/ml, 2.00 times higher than Bvg+, which grew to a maximum density of 2.26×10^9 CFU/ml. This corresponds well with the maximum yield as measured by OD, which was 1.7 times higher in the Bvg- phase.

Measurement of CFU counts revealed that during the later stages of growth there was a loss in cell viability. This occurred in both Bvg phases, and by 66.43 hours the Bvg+ cultures displayed a lower viable CFU count per ml than at the start of growth, while the number of viable bacteria in the Bvg- cultures was more than four times lower than the maximum reached. This loss of viability is something that was not observed when measuring growth by OD, where there was a slight decrease in OD after the maximum yield is reached, but OD remains mostly stable.

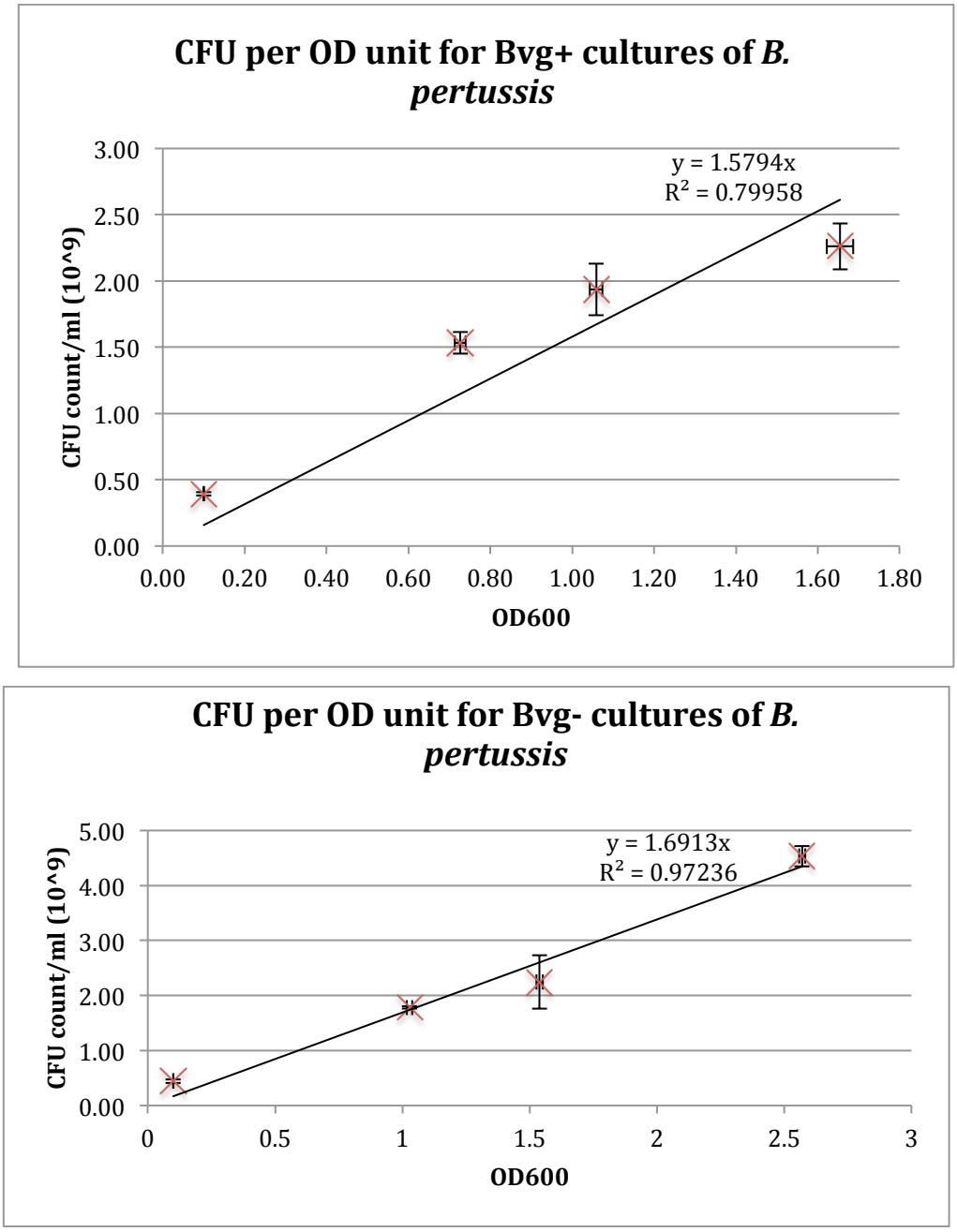


Figure 6- CFU count per ml plotted against OD at time points. As the viable cell count increased the data was plotted against the optical density reading at that time point to discern the relationship between them. A line of best fit was drawn and a good positive correlation can be seen. Data shown for Bvg+ (top) and Bvg- (bottom).

When CFU/ml is plotted against OD for those cultures at the first four time points a linear relationship is shown between the two (figure 6). A strong correlation is observed between CFU/ml and OD showing that regardless of using CFU/ml or OD to measure growth the same trend is observed. As OD doubles, CFU/ml doubles, showing that OD is indeed a good measure of growth of BP536 in SS broth.

However, as noted above, this trend does not hold for stationary phase growth. Measuring growth by OD fails to observe the loss of viability of BP536 cells observed by measuring viable cells counts.

3.1.3 Maximal yields and time taken to reach them

For each of the 11 wells that still contained bacteria at the end of the assay the highest maximum OD_{600} that was measured was noted and an average was taken for Bvg+ and Bvg-. Towards the end of growth, OD readings start to be come variable, especially for the Bvg- phase cultures, as the plate reader is reaching the upper limit of detection. The maximum yields for the Bvg- phase readings should not be the highest points in these spikes, which could represent an artefact of measuring with the plate reader. Thus the maximum yield has been calculated by finding the highest OD reached and averaging this reading together with the four readings either side (representing an hour either side). This averaged value is taken as the maximum OD reached for that culture as it represents a "middle value" between spikes of readings. Values were obtained for each of the Bvg+ and Bvg- cultures and averaged (figure 7).

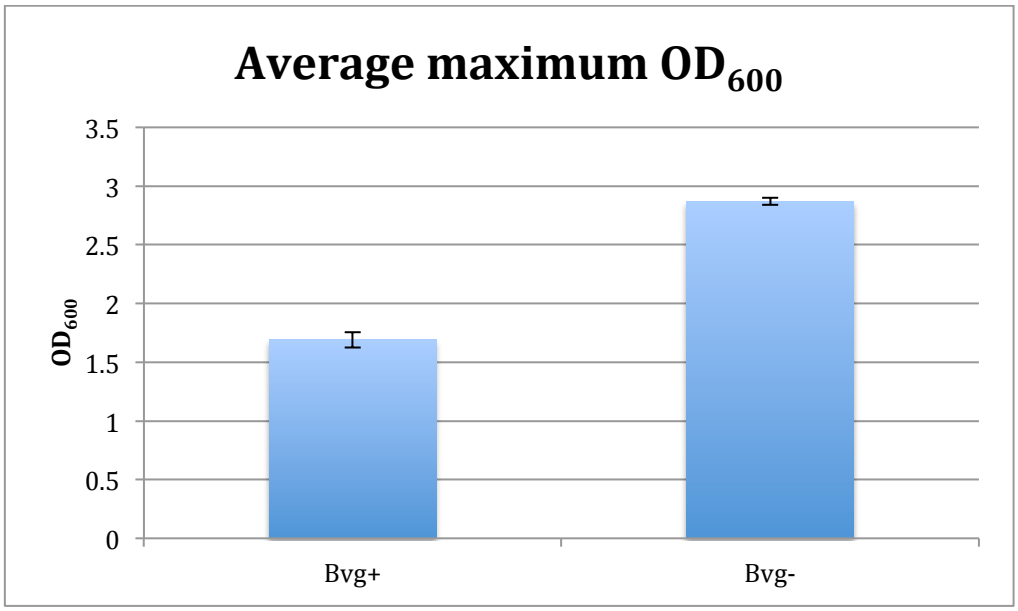


Figure 7- Average maximal OD_{600} reached by eleven Bvg+ and Bvg- cultures. The maximum optical density reached is displayed for Bvg+ and Bvg- cultures (average of eleven biological replicates from within the same 96-well plate). A significantly higher maximum yield is shown for cultures in the Bvg- phase $p=1.64x10^{-10}$. Error shown is standard error of the mean.

The average maximal OD reached by Bvg+ cultures was 1.69, while the average maximal OD achieved by Bvg- cultures was 2.87, 1.70 times higher. It is clear that cultures in the Bvg- phase reached a higher cell density than in the Bvg+. One question that arises is whether or not *B. pertussis* achieves its maximal yield at the same time in each Bvg phase i.e. the Bvg- phase reaches a higher maximal yield than the Bvg+, but does reach it more quickly?

To answer this question the time taken for each of the eleven cultures to reach their maximal yield was recorded and averaged for the Bvg+ and Bvg- cultures (figure 8).

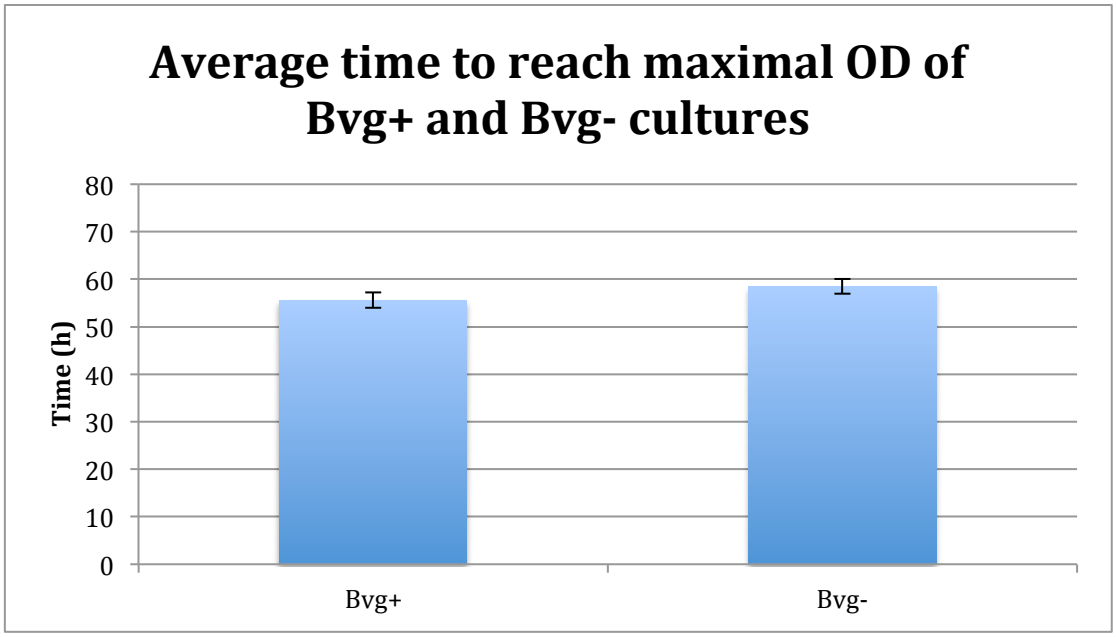


Figure 8- Time taken by eleven BP536 cultures to reach their maximal yield in Bvg+ and Bvg- phase. The time taken to reach maximum yield is expressed in hours and is displayed for an average of 11 biological replicates of Bvg+ and Bvg- cultures from within the same 96-well plate. There is no significant difference between the two averages (p=0.22). Error shown is standard error of the mean.

The average time taken for the Bvg+ cultures to reach their maximal OD was 55.6 hours, while for the Bvg- cultures this was 58.5 hours, however this difference was not significant by T-test (p=0.22). Therefore there was no significant difference in the time taken for cultures to reach their maximal yield dependent on Bvg phase. This means that the maximum yield was reached at the same time regardless of Bvg phase.

It is clear that in the Bvg- phase *B. pertussis* grows to a higher maximum cell density, around double that of Bvg+ phase *B. pertussis*. However, since the bacteria

reach their maximum yields at around the same time, this indicates that the higher yield of the Bvg- phase is not because these cultures grow for longer. Since both phases take the same amount of time to reach very different yields, it is clear that the Bvg- phase cultures must grow quicker in that same space of time to reach a higher yield than the Bvg+ cultures.

Incidentally, the Bvg+ *B. pertussis* finish growing at a lower cell density, and they reach their maximal growth in the same time as the Bvg- phase cultures. The reason why the Bvg+ cultures don't reach the same cell density as the Bvg- phase ones is because they grow at a slower rate in the same time period.

3.1.4 Lag time

A long lag phase is a feature of growth of *B. pertussis*, though it is not clear why this is. It is difficult to say precisely when lag phase ends. All of the cultures undergo a brief, small decrease in OD early in growth, before recovering to the starting OD of 0.1 and beginning to grow. The point at which lag time was designated to be over was set at an OD of 0.3, three times the starting OD, but just before exponential growth had begun. The time taken for each of the eleven cultures to reach this level in each Bvg phase was recorded (figure 9).

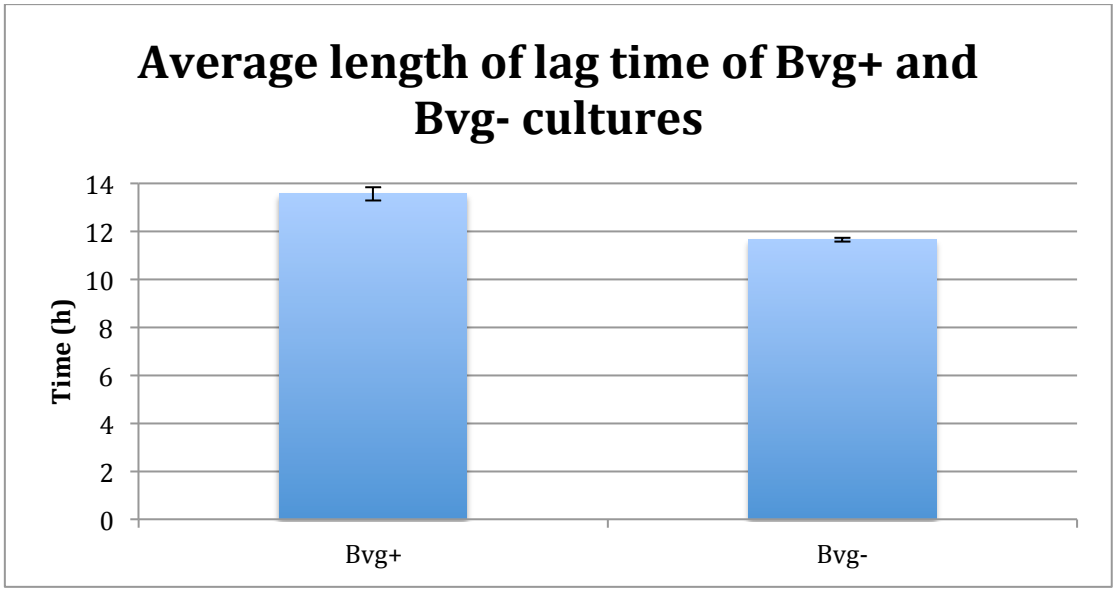


Figure 9- Average length of lag time of Bvg+ and Bvg- cultures. Lag phase was taken as the time in hours of each culture (n=11 of Bvg+ and Bvg-) to reach an OD of 0.3, error shown is standard error of the mean. The average lag phases of Bvg+ and Bvg- phase cultures are significantly different ($p=3.07 \times 10^{-5}$).

The average length of the lag phase of Bvg- cultures was 11.7 hours, while the length of the lag phase of Bvg+ cultures was 13.6 hours, 1.9 hours and 14% longer. This difference was shown to be significant by T-test.

This small but significant difference may not be enough to explain the observation that Bvg+ and Bvg- cultures took the same amount of time to reach different maximal yields, and another factor might also be involved. Bvg- cultures do indeed have a shorter lag phase but it is possible that this difference of two hours is not enough to explain the double maximal yield seen for Bvg- compared to Bvg+. Therefore it could be that growth rate could explain the difference in yield as well as the fact that Bvg- cultures enter exponential phase earlier.

However, the shorter lag phase in Bvg- phase may reveal something about growth in this phase. It is not known why *B. pertussis* has a long lag phase. It may be due to having to synthesise cellular building blocks in order to commit to growth, and it could be that the Bvg- phase is able to do this quicker. An alternative theory is that *B. pertussis* takes some time to adapt to the new medium, and indeed there is a decrease in OD during lag phase before a recovery. It may be that Bvg- phase, is either more more resistant to something in the medium that is initially inhibitory or that this phase is just to better able to adapt to start growing in its new environment.

3.1.5 Growth rates and doubling times

Specific growth rate was calculated for the eleven cultures of BP536 grown in the Bvg+ and Bvg- phases for the time period from 14-42 hours. This period represents the exponential phase of growth in that below 14 hours and above 42 hours growth rate rapidly diminishes. Specific growth rate was calculated using the following equation:

$k = (\log Nt - \log N0)/((\log 2)t)$

Where Nt= final OD in a given time period, N0= initial OD for a given time period, t= the number of hours in a given time period

Which was derived thus:

$Nt = N0 \times 2^n$ (where n= number of generations in a given time period)

Taking logs:

$\log Nt = \log N0 + n\log 2$

Rearranging:

$n = (\log Nt - \log N0)/\log 2$

And:

$n = (\log Nt - \log N0)/0.301$

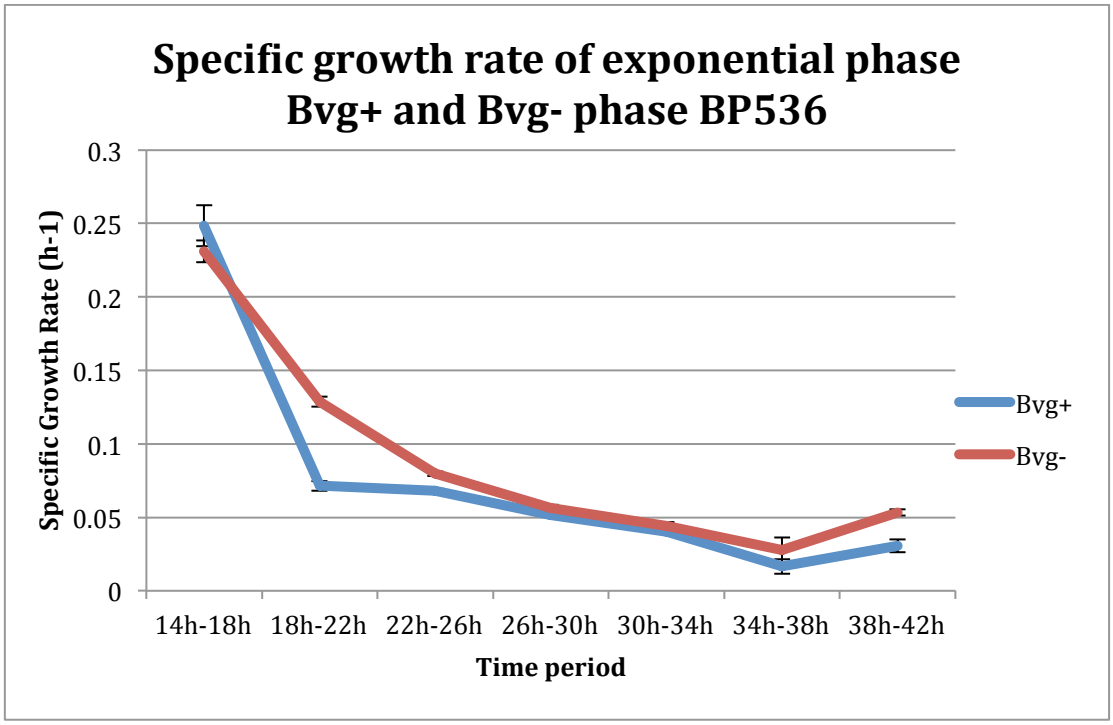
Since k=n/t (specific growth rate is the number of generations per time):

$k = (\log Nt - \log N0)/(0.301t)$

Since the specific growth rate is the number of generations per unit time, the amount of time per generation is calculated by inversing this:

$g = 1/k$

Where g= generation (doubling) time.



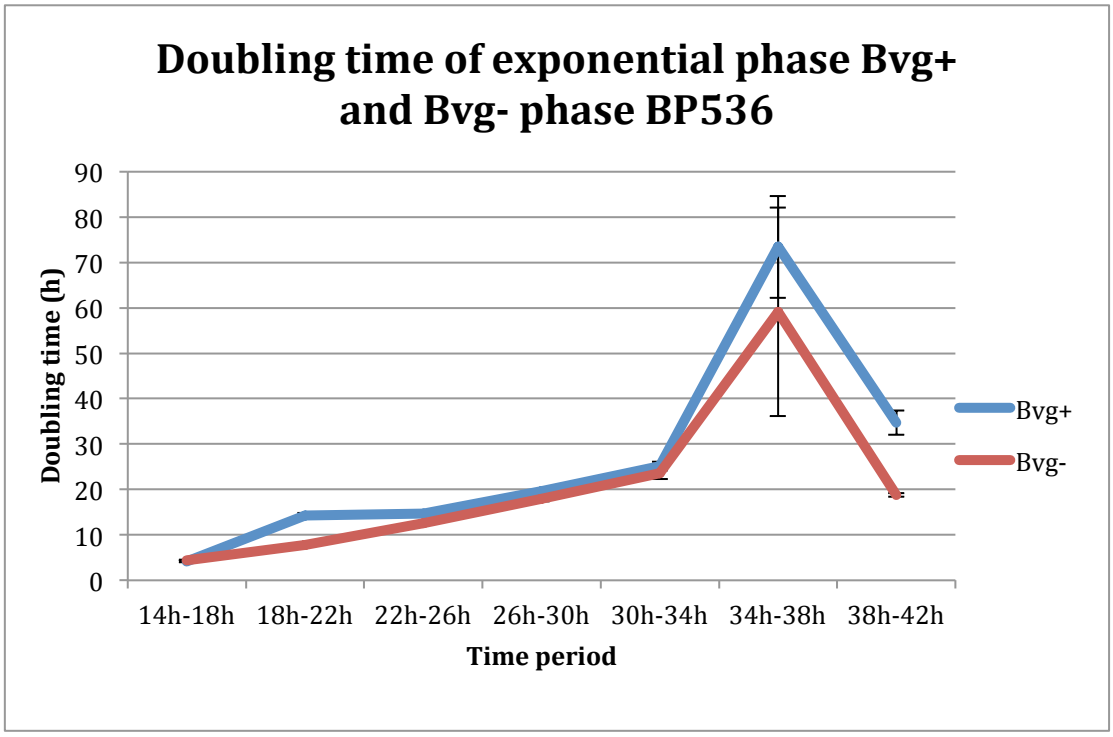


Figure 10- (a) Average specific growth rates and (b) doubling time of BP536 cultures calculated for each four hour period of exponential phase growth. Specific growth rates and doubling times for 11 biological replicates for each Bvg+ and Bvg- cultures from within the same 96-well plate were calculated for each 4 hour intival during exponential phase. Data is an average of the 11 replicates; error shown is standard error of the mean.

Figure 10 (a) shows that the average specific growth rate of both Bvg+ and Bvg- BP536 between 14 and 18 hours is $0.24h^{-1}$. This corresponds to a doubling time of 4.4 hours (figure 10 (b)). For both Bvg+ and Bvg- this is the maximal growth rate. The main difference between Bvg+ and Bvg- phase growth is how quickly this maximal growth rate decreases. For the next time period, 18-22 hours, the average specific growth rate for the Bvg+ cultures is 0.0714 h^{-1}, while for the Bvg- cultures are at 0.129 h^{-1}, which is 1.8 times higher than for Bvg+ ($p=1.30x10^{-10}$). The corresponding doubling times for Bvg+ and Bvg- for this time period are 14.3 h and 7.82 h respectively. The average doubling time of the Bvg- cultures is 1.8 times higher ($p=2.08x10^{-7}$). The average specific growth rates decrease again during the next time period (22-26 hours) to 0.0681 h^{-1} and 0.0797 h^{-1} ($p=2.53x10^{-5}$) for the Bvg+ and Bvg- cultures respectively. For the time period 26-30 hours the average specific growth rates for Bvg+ and Bvg- cultures is 0.0514 h^{-1} and 0.0563 h^{-1} ($p=0.0561$). The average doubling times during this period are 19.6h for the Bvg+ cultures and 18.0 for the Bvg- cultures ($p=0.0428$). For the remainder of exponential phase there is no significant difference between Bvg+ and Bvg- cultures with respect to specific growth rate or doubling time.

Therefore the main difference in regard to growth rate is that while maximal growth rate is similar between Bvg+ and Bvg- cultures, and this is not maintained for long in either phase, the growth rate decreases more rapidly in the Bvg+ phase than the Bvg- phase.

3.1.6 Dry cell weight

The dry cell weight of flask cultures was measured over time to provide information about how cell mass increased during growth of a culture. The results are depicted in figure 11.

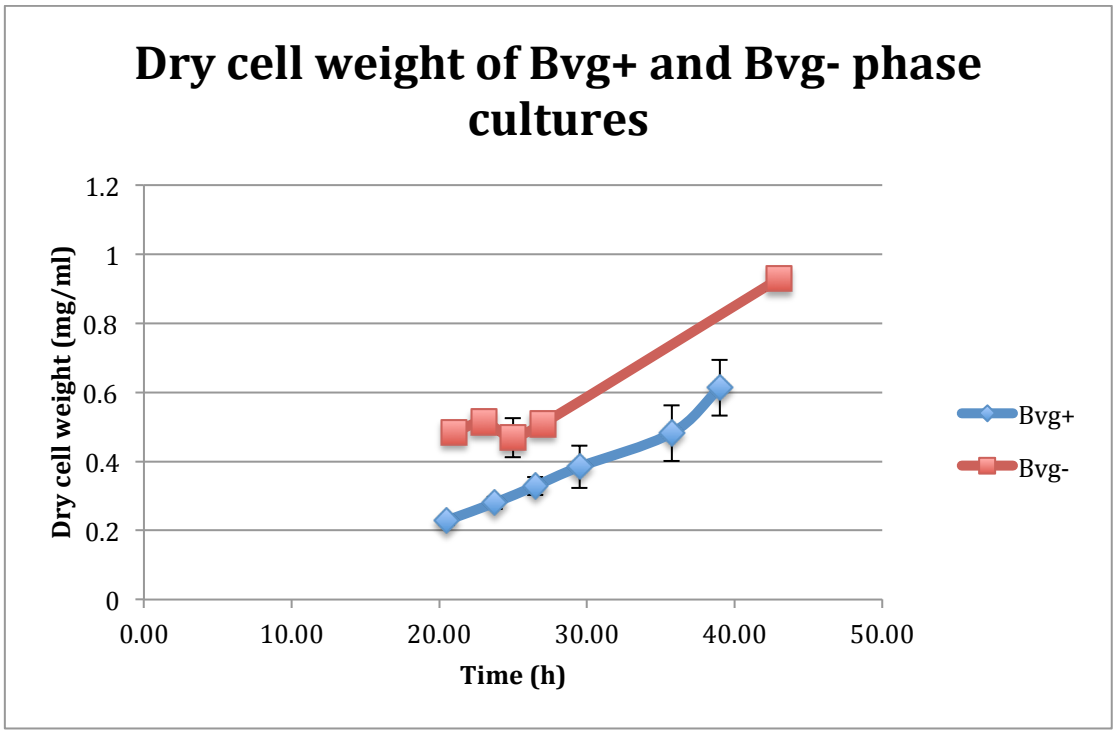


Figure 11– Dry cell weight per ml of culture measured over time for BP536 in the Bvg+ and Bvg- phases. The dry cell weight of 3 biological replicates of Bvg+ and Bvg- cultures grown in flasks was measured over time. Data shown is an average of the three replicates and error is standard error of the mean. Dry cell weight is measured in mg of dry cell weight per ml of culture.

In both Bvg phases of growth the dry cell weight of cultures measured in mg per ml of culture increases over time and at every point sampled the Bvg- cultures have a greater mass per ml than the Bvg+ cultures. This is expected since it was shown above that growth in the Bvg- phase produces more dense cultures than the Bvg+ cultures, as measured by OD and CFU count. Therefore Bvg- cultures are of greater cell density to Bvg+ cultures and weigh more. However, an important question to ask is how much more do they weigh? It would be expected that a culture with double the number of cells would have double the mass if the cells in each culture were the same size. If cell density in OD is plotted against dry cell weight in mg/ml then a relationship between the two can be seen.

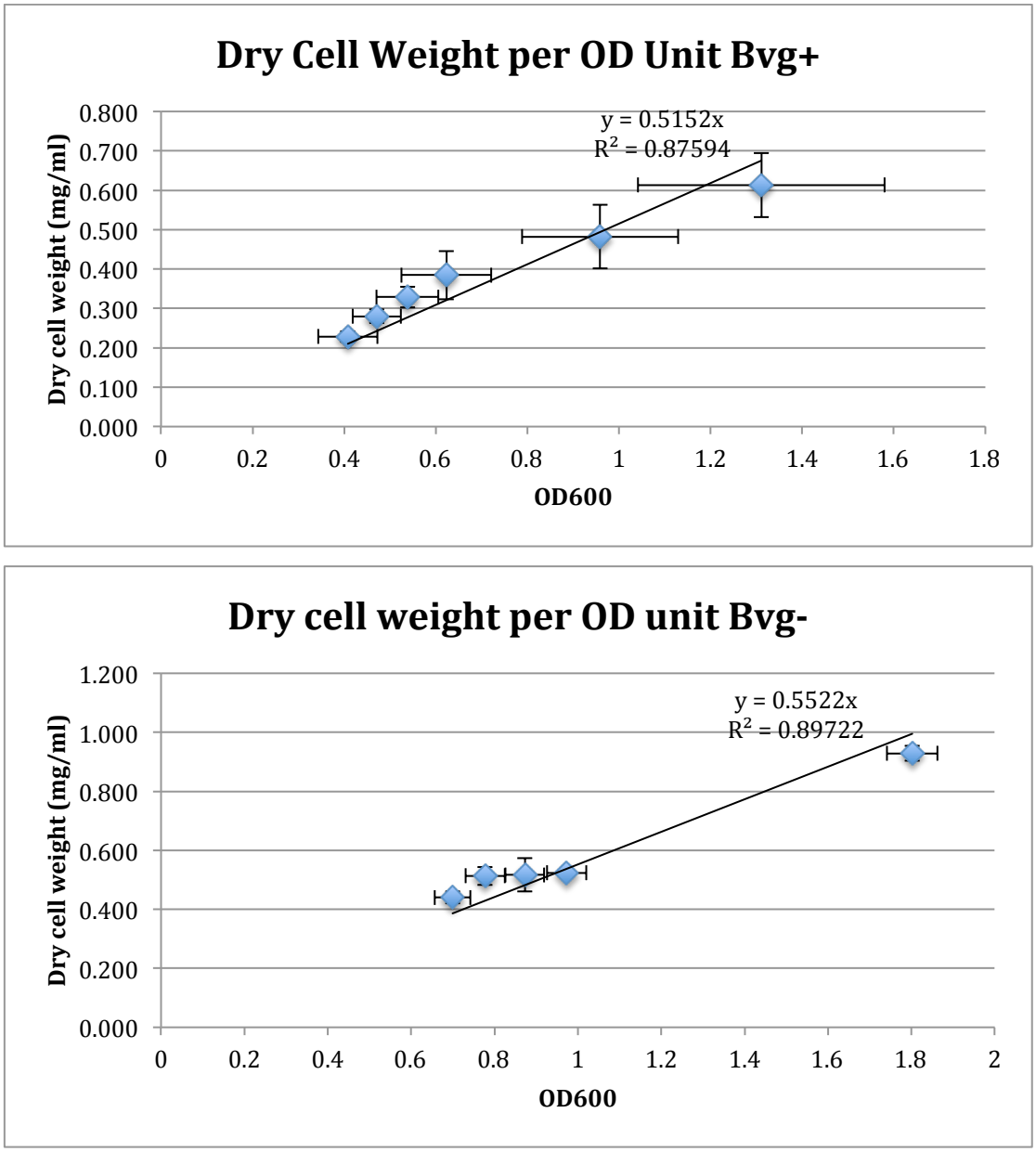


Figure 12– Dry cell weight of cultures plotted against OD for cultures of BP536 in the Bvg+ (top) and Bvg- (bottom) phases. The data from dry cell weight measurements is plotted against the optical density measurements at equal time points. For both Bvg+ and Bvg- cultures a linear relationship can be drawn between the two such that as dry cell weight increases so does optical density in a proportional manner. Data shown is the average of three replicates and error is standard error of the mean.

A linear relationship can be drawn between the dry cell weight of a culture and OD in both Bvg phases (shown in figure 12). The equations of the line of best fits for each phase are similar and can be used to work out that 1 ml of a culture of OD 1.0 would weigh 0.515 mg grown in the Bvg+ phase and 0.552 mg in the Bvg- phase. This information, together with the observation above that cultures at OD 1.0 have the

same numbers of cells regardless of Bvg phase, shows that biomass, OD and CFU count of BP536 cultures increase in the same way throughout exponential phase.

3.1.7 Glutamate consumption

The concentration of glutamate was measured in the supernatant that was removed from samples taken to obtain dry cell pellets. This was done in order to measure the rate of glutamate consumption for growth of BP536. The amount of glutamate in millimoles consumed to make one gram of biomass during exponential phase growth is displayed in figure 13.

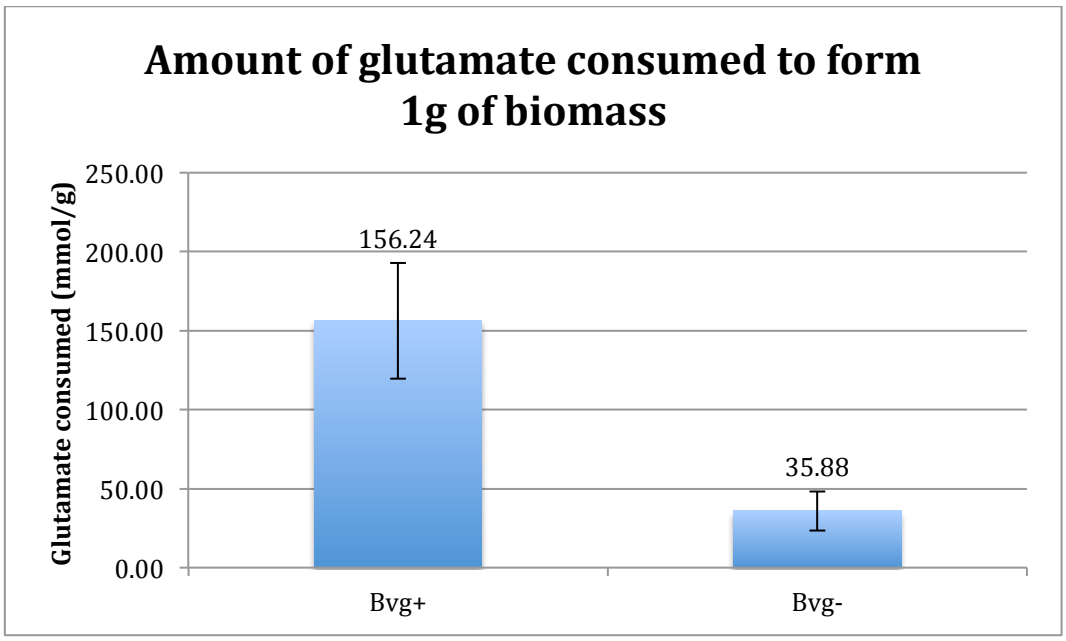


Figure 13– Amount of glutamate consumed to form 1g of biomass during exponential phase growth of BP536 in the Bvg+ and Bvg- phase. Glutamate consumption between two time points during exponential phase was calculated for 3 biological replicates of both Bvg+ and Bvg- cultures and expressed as the amount of glutamate in mmol consumed to produce 1 gram of biomass (mmol/g). Data shown is the average of three replicates; error is standard error of the mean.

The data show that Bvg+ phase cultures are consuming 4.4 times more glutamate to produce one gram of biomass than Bvg- phase cultures. This suggests that, since more carbon is being consumed, the Bvg+ phase cultures are behaving metabolically different to the Bvg- cultures. The data imply that there is extra carbon consumed in the Bvg+ that is not accounted for in biomass and would be lost during growth, either in the supernatant or as waste such as carbon dioxide lost as gas. If more carbon

dioxide is produced in the Bvg+ phase this would be indicative of a more active TCA cycle since it is during this that carbon dioxide is formed.

3.1.8 Measurements of key metabolites

To look for metabolic differences in the growth of BP536 in the Bvg+ and Bvg- phases the concentrations of key metabolites in the supernatant during growth were measured. This included the primary carbon source glutamate and ammonium, fatty acids and beta-hydroxybutyrate (β-HB) that are known to accumulate during growth.

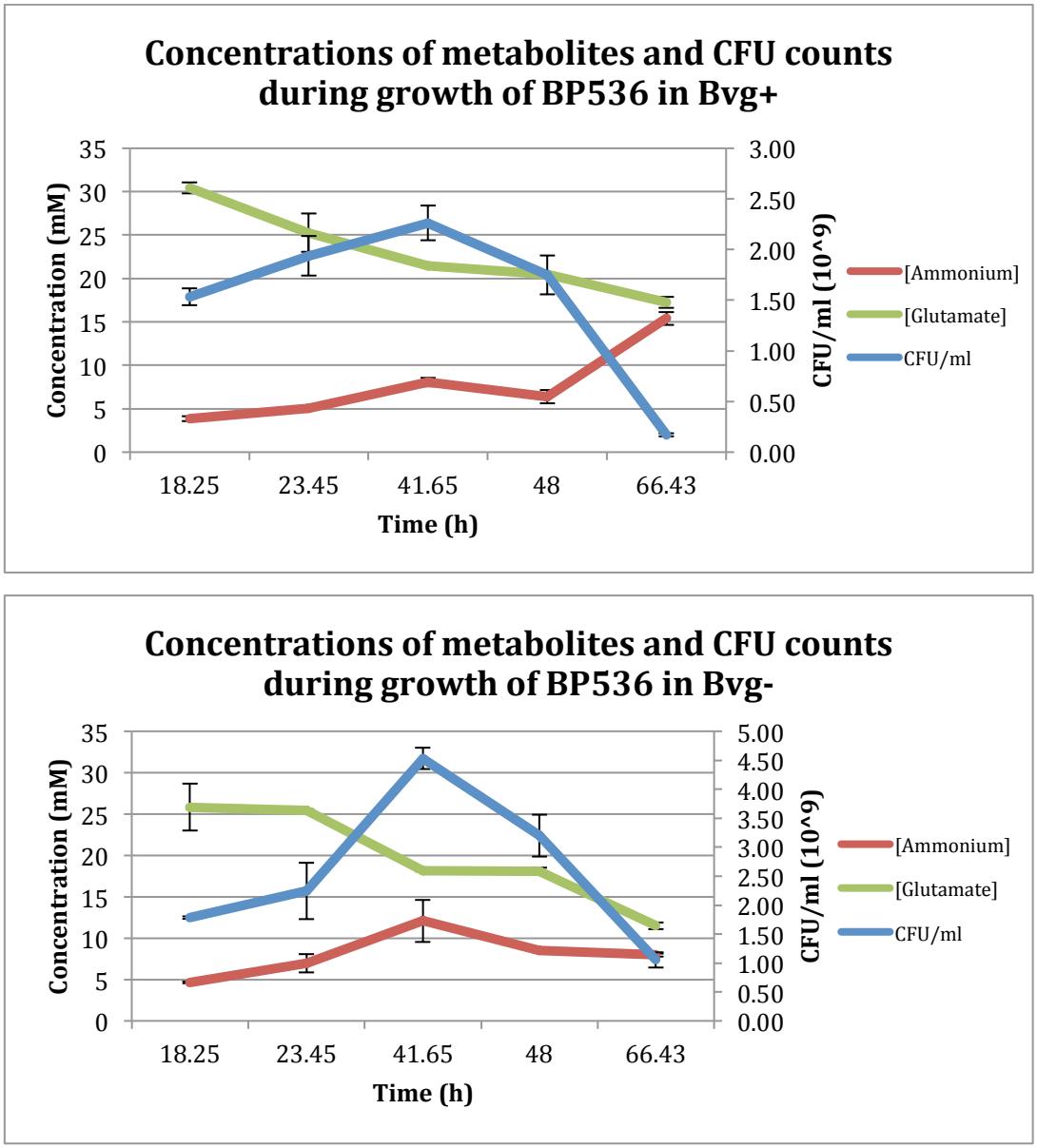


Figure 14- Concentrations of ammonium and glutamate (left axis) with CFU count per ml (right axis) during growth of BP536. Measurements of glutamate and ammonium concentration were taken throughout exponential and stationary phase growth of cultures grown in the Bvg+ (top) and Bvg- (bottom) phases. Data shows concentrations of these molecules in mM over time and is displayed together with CFU data of cultures to show how viable cultures were during these measurements.

Figure 14 shows that as viable cell counts increase glutamate is consumed from the medium. The utilisation of glutamate coincides with the appearance of ammonium. These processes occur in both Bvg phases, though the final concentration of ammonium is higher in the Bvg+ phase (15.4mM) than in the Bvg- phase (7.96mM), despite the final concentration of glutamate in the supernatant being higher in the

Bvg+ phase (17.2mM) than in the Bvg- phase (11.5mM). This indicates that BP536 may produce less ammonium per unit of glutamate consumed in the Bvg- phase.

CFU counts reach a peak at 41.65 hours in both Bvg+ and Bvg- phase, after which viability counts decrease. However, glutamate concentrations in the supernatant continue to decrease during this period of decrease in viability counts. This suggests that cells are still dividing, utilising glutamate, but the rate of cell death is greater than the dividing rate. Alternatively, cells could be non-dividing, but still consuming glutamate to maintain themselves, at the same time as cells dying.

Furthermore, glutamate remains in the medium at millimolar concentrations after 66.43 hours of growth, suggesting that cultures don't cease growing because of lack of availability of carbon source. In fact, the observation that glutamate continues to be consumed past the peak of cell density suggests that growth yield is not limited by glutamate availability.

The build-up of ammonium could be the reason for loss of viability and indeed the decrease in cell viability does coincide with the accumulation of ammonium. The concentration of ammonium at peak growth is 8.05mM in the Bvg+ phase and 12.09mM in the Bvg-, both more than double the concentration at the first measurement at 18.25 hours. Since this build-up of ammonium coincides with peak growth in both phases it is tempting to suggest that this is the reason why growth ceases. After this the concentration of ammonium in the Bvg+ phase cultures continues to increase, while the concentration in the Bvg- phase cultures decreases slightly. It may be that something else contributes to loss of viability of BP536 during growth. Build-up of free fatty acids is also known to be inhibitory to growth, but concentrations of these were always below the limit of detection using the commercially available kit.

During growth PHB is known to accumulate intracellularly and is broken down and secreted as the monomer β-HB. This is primarily a mechanism of recycling acetyl-CoA for feeding into the TCA cycle, but β-HB is essentially a waste product. The concentrations of β-HB in the supernatant were measured during growth.

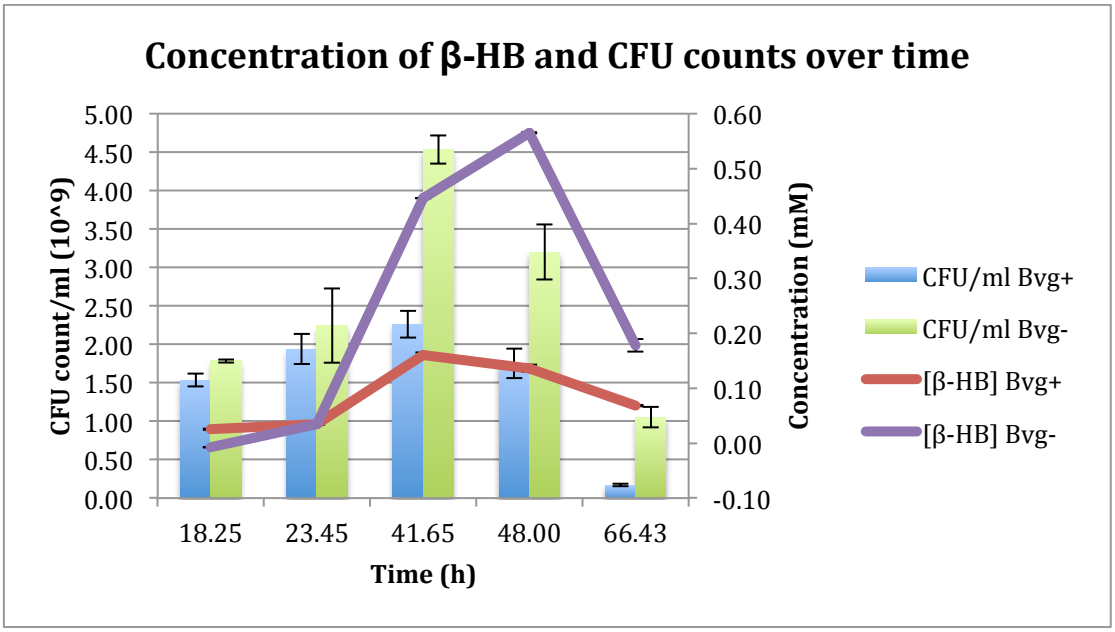


Figure 15- Concentrations of β-HB (right axis, lines) with CFU count per ml (left axis, bars) during growth of BP536 in the Bvg+ and Bvg- phase Concentrations of β-HB were measured at time points during exponential and stationary phase for both Bvg+ and Bvg- phase cultures and is expressed in mM. This data is displayed along with CFU counts for cultures during the same time periods expressed as CFU counts/ml to show how the concentration of β-HB changed during growth of cultures**.**

Figure 15 shows that β-HB appears in the medium during growth, but begins to disappear later on. The maximum concentration of β-HB measured is 0.161mM for Bvg+ phase reached after 41.65 hours, and 0.565mM reached after 48 hours for the Bvg- phase. After this the concentrations decrease despite a decrease in viability counts, suggesting that β-HB may be metabolised.

3.1.9 Build-up of ammonium and β-HB

The build-up of ammonium was assessed between the time at which the first sample was taken and the time at which the growth was beginning to cease. The time at which this sample was taken was at 41.67 hours. The difference in ammonium concentration between these two times points was divided by the difference in OD_{600} between these two time points. This gives a measure of ammonium build-up over time corrected for the increase in growth. This is displayed in figure 16.

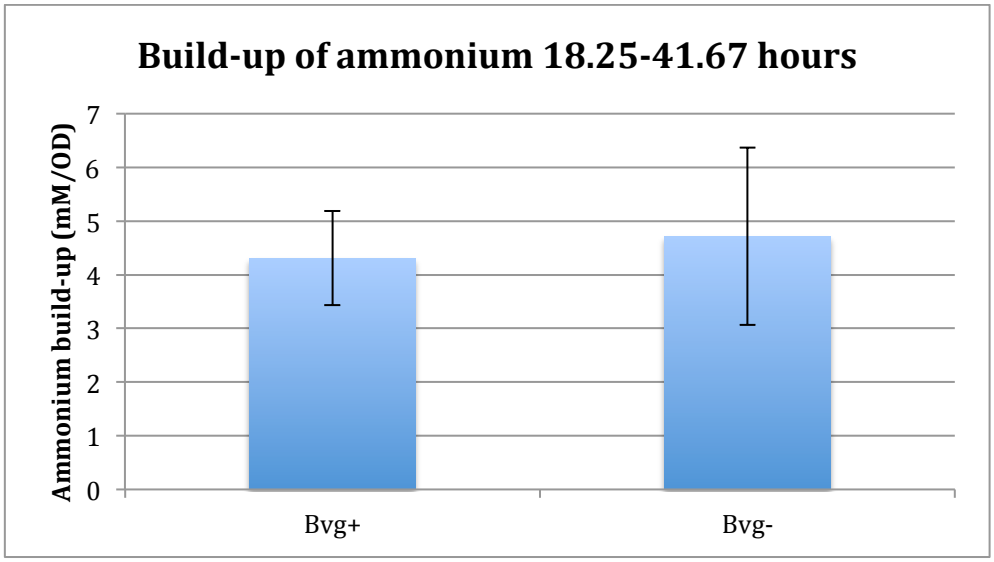


Figure 16- Build-up of ammonium per OD unit produced during the growth of Bvg+ and Bvg- cultures between 18.25 and 41.67 hours. The difference between the concentrations of ammonium at 41.67 hours and those at 18.25 was calculated and divided by the increase in optical density during the same time period. Therefore data shown is representative of the build-up of ammonium per growth unit between these time periods expressed as mM of ammonium produced per OD unit. Data shown is an average of three replicates and error is standard error of the mean.

Figure 16 shows that between these 18.25 and 41.67 hours there is no difference in the amount of ammonium that has accumulated during growth in the Bvg+ and Bvg- phases when corrected for growth differences. The build-up was calculated between these time points because this is the point at which growth is beginning to cease, and if ammonium is inhibiting growth it would be expected that inhibition would take effect at around this time point. The build-up of ammonium was also calculated between 18.25 and the end of the growth assay, at 66.5 hours. This data is presented in figure 17.

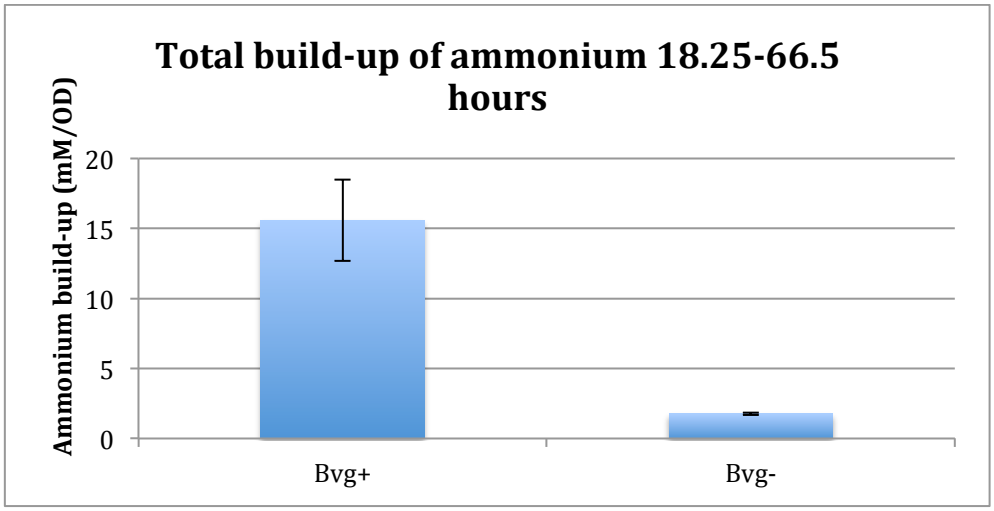


Figure 17- Build-up of ammonium per OD unit produced during the growth of Bvg+ and Bvg- cultures between 18.25 and 66.5 hours. Data was calculated in a similar fashion to that displayed in figure 16 except for a different period of time. Data shown is representative of the build-up of ammonium per growth unit between 18.25 and 66.5 hours expressed as mM of ammonium produced per OD unit. Data shown is an average of three replicates and error is standard error of the mean.

The build-up of ammonium per OD unit is significantly greater in the Bvg+ phase than in the Bvg- phase over the whole of the growth assay ($p=0.041$). This shows that later on, after growth has ceased (i.e. between 41.67 and 66.5 hours) the Bvg+ continues to produce ammonium, while the Bvg- phase does not.

The build-up of β-HB was assessed between 18.25 and 48 hours, when the concentration of β-HB was at the maximum.

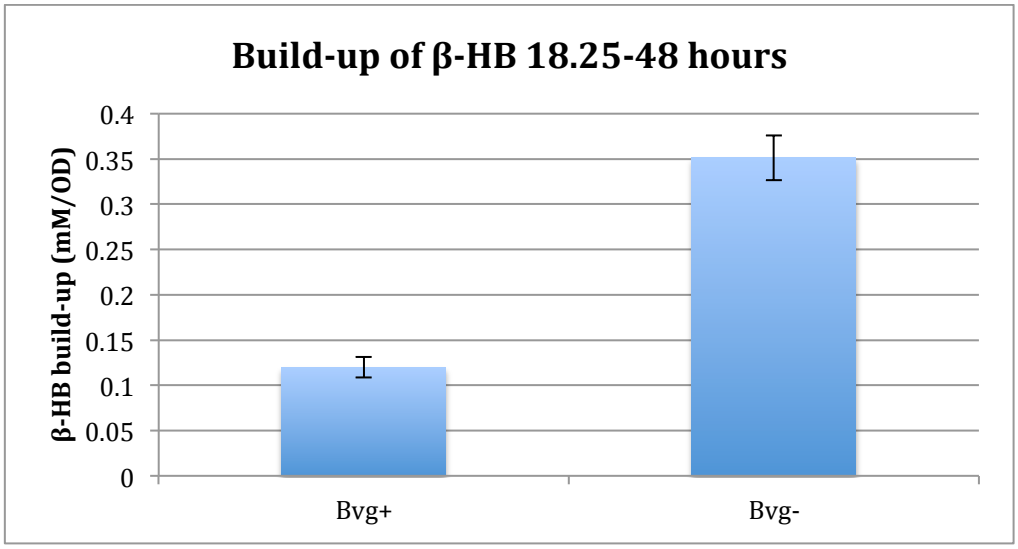


Figure 18- The concentration of β-HB in the supernatant corrected for OD of the cultures betwwen 18.25 and 48 hours of growth for Bvg+ and Bvg- cultures. The difference between the concentrations β-HB of at 48 hours (taken to be the time at which β-HB levels were maximal) and concentrations at 18.25 was calculated and divided by the increase in optical density during the same time period. Therefore data shown is representative of the build-up of β-HB per growth unit between these time periods expressed as mM of β-HB produced per OD unit. Data is the average of three replicates; error is standard error of the mean.

Figure 18 shows that there is a significant difference in the concentration of β-HB secreted into the medium per OD unit between Bvg+ and Bvg- cultures ($p=0.043$). During this period of growth the Bvg- cultures produce almost three times as much β-HB as Bvg+ cultures when higher growth yields are taken in to account.

3.2 Discussion

3.2.1 Measuring cell growth

Optical density is a way of indirectly measuring growth of a culture by measuring the scatter of light caused by an increase in turbidity of the culture during growth. This increase is not necessarily specific to growth and is affected by other factors including the size of particles scattering the light, or colour changes. A more specific way to measure culture growth is by CFU count, which not only gives a measure of number of cells in the culture but also that they are viable. The advantage of measuring growth of a culture by OD is that it can be an automated process, particularly

advantageous if the growth of the culture is to be measured over period of days, as with *Bordetella pertussis*.

Growing cultures in a 96-well plate allows for a higher-throughput, automated method of measuring growth of *B. pertussis* than would otherwise be possible manually. Many replicates can be measured simultaneously allowing for reproducible growth of organisms that can be variable in their growth, such as *B. pertussis*. This method also allowed for the ability to sacrifice up to four cultures at a time throughout growth to measure concentrations of metabolites.

It is not known whether *B. pertussis* has different cell sizes dependent on growth in Bvg+ or Bvg- phase, which may affect OD measurements. Therefore, whilst carrying out an automated growth measurement of OD it is necessary to measure growth another way, for example, taking samples for CFU measurements, which also gives information about the viability of cells in the cultures. Since OD is more of an accumulative measure of growth than CFU counts, it does not give information about cells that may be dead but intact. Similarly, measuring by CFU count gives information about the number of viable cells in the culture, but does not detect cells that may be alive but non-culturable. It is desirable then, when describing growth of an organism in detail, to provide both measurements, as one method may uncover something that would be missed by the other.

Growth of BP536 in the Bvg+ and Bvg- phases here was principally measured by OD as it is the most automated method, but CFU counts were used to validate indirect measurement by OD, i.e. does a doubling of OD correspond to a doubling of CFUs? A direct relationship was shown between CFU count and OD for BP536 in both Bvg phases, showing that measurement by OD is a valid way to measure growth of *B. pertussis* in a way that allows for comparison of the Bvg+ and Bvg- phases.

However, the two methods do reveal different aspects of the growth of *B. pertussis*. Measuring by OD shows an exponential increase to a maximum and bar a slight decrease in OD over stationary phase, maximal OD is maintained. Measuring by CFU count shows a different picture, maximum growth is reached in the same time period as shown with OD measurements, however maximum cell viability is not maintained, with CFU count rapidly decreasing in both Bvg phases following the maximum yield.

Two scenarios are possible. The first is that increasing numbers of cells in stationary phase are dead, but OD measurements fail to reflect that, while the true

number of viable cells is measured by CFU counts. The second is that cells in stationary phase are alive, but are non-cultureable or metabolically inactive, which is why they are not cuturable on agar plates.

3.2.2 Growth yield of Bvg- phase *B. pertussis*

Analysis of growth of *B. pertussis* by OD allows for automation of the process, meaning that growth can be sampled every fifteen minutes over a period of days. This also gives a high resolution to the growth curve, which means that growth can be broken down and analysed in detail.

One of the most striking difference between Bvg+ and Bvg- phase growth is that the Bvg- phase gives growth yields 1.7 times that of the Bvg+ phase. This shows that cell density is significantly higher than that of Bvg+ phase in the Bvg- phase, despite the Bvg- phase growing to the upper limit of detection by the plate reader used to measure OD. Maximal yield of a culture can be affected by many things, among them growth rate and the length of growth. Cultures could divide more often in a given time to reach a higher OD, or cultures could divide at the same rate but if one grows for longer it will reach a higher OD. The finding that BP536 reaches its maximal OD in the same time regardless of Bvg status shows that the Bvg- phase reaches a higher OD for a reason other than because these cultures grow for longer. It has been postulated that *B. pertussis* fails to reach higher yields because of the build-up of autoinhibitory compounds such as ammonium and free fatty acids [120, 122]. If the Bvg- phase was more tolerant of these compounds it might be expected that the Bvg- phase would continue to grow past the point at which the Bvg+ phase stops growing, and that is not the case. Therefore something other than resistance to autoinhibitory compounds, or the Bvg- phase producing less of them, is responsible for the Bvg- phase reaching higher yields.

A feature of *B. pertussis* growth is a long lag phase (between 11 and 13 hours). The reasons for this are unknown, though it would seem that this is not due to adaptation to medium since the lag phase is replicated on passage in fresh medium (the cultures used for the growth assay depicted in figure 4 had been grown in SS broth previously). It could be that there are elements in fresh medium that are inhibitory for growth, or that there are molecules that need to be synthesised to a threshold level before *B. pertussis* commits to growth, though both of these scenarios

would seem to be adaptation in some way. Alternatively, there could be components of the medium that need modifying before growth can occur. What is clear is that Bvg- phase growth displays a shorter lag phase than Bvg+, which may be indicative of the ability to synthesise necessary molecules faster, or being more resistant to inhibitory molecules in the medium.

Furthermore, while maximal growth rates are not maintained in either Bvg phase, higher growth rates are maintained for longer in the Bvg- phase. This would mean that early fast growth is crucial for and predictive of a high final yield. The reason for a higher growth rate in the Bvg- phase is unclear, although it is suggestive of a difference in regulating cell division. Typically growth rate is improved by growing cells in richer media, or by genetically manipulating organisms to improve flux through targeted pathways, but in this case the only difference is Bvg status. In other words Bvg is a switch that controls how quickly cells divide.

Different models have been suggested for what controls when a cell divides. One suggests that all cells grow a certain amount before dividing, while another proposes that all cells grow to a certain length [150, 151]. It is not clear what triggers *B. pertussis* cells to divide, a set cell size or a set amount of growth. If all *B. pertussis* cells grew to a predetermined length then cells in the Bvg- phase must grow quicker since they divide more often, unless cells in the Bvg- phase are smaller. Likewise, if *B. pertussis* cells grow by the same amount before dividing, then cells in the Bvg- phase must also grow quicker. The data obtained from biomass measurements and the relationship between them, OD and CFU show that a given volume of a given OD of cells contains similar numbers of cells and has a similar dry cell weight regardless of Bvg phase. This suggests that, since the same number of cells weighs the same in either Bvg phase that the size of the cells is also the same. This would indicate then that in the Bvg- phase cells would grow quicker to reach the same size or growth threshold as in the Bvg+ phase before dividing.

3.2.3 Glutamate

Glutamate is consumed during growth, and is consumed past the point of peak growth yield. This shows that in stationary phase cells are still metabolically active despite no net growth of the culture. The reason why there is no net growth at this stage is not clear, as there are still millimolar levels of glutamate left in the medium. This points

to inhibitory compounds preventing growth at this point, and the observation that Bvg+ and Bvg- phase cultures enter stationary phase suggests that if they do stop growing because of inhibitory compounds then these compounds affect cultures regardless of Bvg phase.

It is not known, however, that the concentration of glutamate during stationary phase is enough to support growth, especially at high cell densities. It may be that at such cell densities the amount of glutamate per cell is enough to give a level of glutamate transport across the membrane to support some metabolic activities but not cell division. This would explain why glutamate continues to be consumed during stationary phase despite no net increase in cell number.

During growth *B. pertussis* consumes more glutamate per unit of growth in the Bvg+ phase than in the Bvg-. This means that more glutamate is being consumed to make the same amount of biomass, in the Bvg+ phase and gives rise to the question of where the extra carbon being consumed is going.

The obvious answer is that more carbon is needed by the Bvg+ phase to produce the virulence factors expressed in this phase, including large adhesins such as FHA and fimbriae, however some (but not all) of these proteins would be included in biomass measurements and are accounted for when calculating glutamate consumption. DNA and RNA would also be accounted for in biomass, as would carbohydrates although one might expect more carbohydrates to be made in the Bvg- phase since it is in this phase that the capsule is expressed.

Carbon dioxide is produced during metabolism and it stands to reason that if Bvg+ phase cultures are consuming more glutamate to produce less biomass then this extra carbon that wouldn't be accounted for in biomass measurements is released as carbon dioxide gas. This would suggest a more active TCA cycle since it is this process that leads to the production of carbon dioxide. This would lead to an increase in the level of NADH for use in the electron transport chain compared to the Bvg- phase.

3.2.4 Ammonium

It has been suggested that since ammonium accumulation coincides with cessation of growth, that ammonium is inhibitory for growth [122]. It has also been shown that growth can be improved by replacing some of the glutamate in the medium with

lactate, with the result that less ammonium is produced [122]. However, it is not clear whether ammonium is actually toxic for growth of *B. pertussis*, or whether its appearance alongside the slowing of growth is coincidental. It has been shown for other bacteria that concentrations of ammonium in the region of 500mM is inhibitory for growth, but that this inhibition is not ammonium specific but rather a consequence of enhanced osmolarity or ionic strength of the medium [152]. The maximum concentration of ammonium produced during growth of *B. pertussis* is 15mM, a lot less than 500mM, though the level at which ammonium is inhibitory for growth of *B. pertussis* is not known.

B. pertussis produces more ammonium per unit of growth in the Bvg+ phase than in the Bvg- phase over 66 hours. Ammonium is a by-product of glutamate metabolism, specifically the conversion of glutamate to 2-oxoglutarate catalysed by glutamate dehydrogenase (*gdhA*). Since ammonium is produced from the pathway which feeds glutamate into the TCA cycle it means that ammonium is an unavoidable by-product of metabolising this carbon source. It stands to reason that if the Bvg+ phase is metabolising more glutamate per growth unit then it will also produce more ammonium. It would be logical then to assume that since the amount of ammonium per biomass is higher in the Bvg+ phase that this is a reason why growth yield is lower, although the absolute concentrations of ammonium are similar between the two at maximal growth and at the point of cessation of growth the build-up of ammonium in mM/OD unit is similar. It may be that 8-12mM of ammonium is inhibitory for the growth of *B. pertussis*, which would explain why both Bvg+ and Bvg- culture cease growing at the same times, despite millimolar concentrations of glutamate still in the medium.

The concentration of ammonium in the supernatant continues to increase through stationary phase in Bvg+ cultures, which is not the case for Bvg- cultures, in which the ammonium concentration decreases. This may reflect different nutrient requirements of the two Bvg phases later on in growth. Both phases continue to metabolise glutamate, which would mean ammonium would be produced. It could be that the Bvg- phase cultures then use ammonium produced as a nitrogen source. This could be to make a nitrogen-containing compound that isn't made in the Bvg+ phase. An alternate scenario that leads to no net increase in ammonium concentration is one in which there is a build up of an alternative source of carbon to glutamate, one that doesn't lead to an excess of nitrogen. This could be something produced during

growth that can then be used up as glutamate concentrations deplete. Bvg+ phase cultures may continue to use glutamate at an appreciable rate, leading to the continued accumulation of ammonium during stationary phase, while the Bvg- phase uses this alternative carbon source at a greater rate than the Bvg+ phase, leading to a decrease of ammonium concentration in stationary phase. This carbon source would likely not contain nitrogen and ammonium would be used as a source of nitrogen in this case.

3.2.5 β-HB

PHB is known to accumulate in globules during growth of *B. pertussis* in SS broth, which then disappear later on in growth, coinciding with the appearance of β-HB in the supernatant. This is thought to be a mechanism for regenerating CoA from an excess of acetyl-CoA. The first CoA would be released by condensing two acetyl-CoA molecules to acetoacetyl-CoA, also forming β-HB, and the second CoA is released as β-HB is polymerised to PHB. PHB is also broken down during growth, contributing to biomass and also releasing β-HB into the supernatant [122].

During growth of BP536 in both Bvg+ and Bvg- phases there was a build up of β-HB in the supernatant, suggesting that CoA is a limiting factor in both phases of growth, or that acetyl-CoA is in excess. In the Bvg- phase, however, the concentration of β-HB builds up to 3.5 times that of the concentration in the Bvg+ phase. This could be due to one of two scenarios. In the first, more CoA is required for growth, or acetyl-CoA is in greater excess in the Bvg- phase. The Bvg- phase would have more active PHB synthesis pathways, which leads to more β-HB accumulating in the supernatant, while the Bvg+ phase might be able to use acetyl-CoA differently. For example, a more active TCA cycle in the Bvg+ phase would use up more acetyl-CoA by turning it into citrate and feeding the TCA cycle pathways. This would fit with the model that the Bvg+ uses more glutamate to make less biomass than the Bvg- phase and that the excess carbon is being lost as carbon dioxide through a more active TCA cycle.

Another possibility is that PHB is not made any differently and is made at the same rate in both phases. It may be that it is the breakdown of PHB that is different, meaning that more β-HB appears in the Bvg- phase because more of its PHB is broken down. However, it has been shown that PHB does disappear later in growth, as β-HB appears in the supernatant. This does suggest that the amount of β-HB

appearing is proportional to the amount of PHB that was synthesised, which would indicate that if Bvg- phase growth leads to greater concentrations of β-HB then growth in this phase also produced more PHB.

β-HB concentrations in the supernatants of both Bvg+ and Bvg- phase growth decrease during stationary phase, suggesting that β-HB is being metabolised, possibly used as a carbon source. The decrease in the Bvg+ phase was a two-fold drop between 48 hours and 66.43 hours, while in the Bvg- phase during the same time period the decrease was a three-fold drop. During this period there is no net increase in cell number, although glutamate consumption continues. Ammonium levels decrease during this period for Bvg- cultures, but continue to increase for Bvg+ cultures. This suggests that while cells in both cultures continue to be metabolically active, the Bvg- phase metabolises more β-HB than the Bvg+ phase, producing less ammonium since β-HB does not contain nitrogen. This indicates a difference in use of carbon source, it may be that the Bvg+ is not able to metabolise β-HB as well as the Bvg- phase. Why this would happen is not clear, since in both phases cultures do not increase in cell number at this stage of growth, although it may be that glutamate at that concentration is not enough to sustain growth and that metabolising β-HB is a way of keeping cells alive and metabolically active.

3.2.6 A proposed model for growth differences between Bvg+ and Bvg- phase *B. pertussis*

It is clear from growth data that there is something quite different about the way that *B. pertussis* grows that is dependent on Bvg phase. In the Bvg- phase maximal yield is 1.7 times that of the Bvg+ phase, but both phases reach their maximal yields in the same time. Growth in both phases is characterised by slowing of growth rate throughout exponential phase, though during early exponential phase the growth rate of the Bvg- phase is significantly higher than the Bvg+ phase. Since the Bvg- phase has a shorter lag phase and has a higher growth rate it could be that these features are linked, with the Bvg- phase better able to synthesise molecules early on to commit to growth, while the Bvg+ phase takes longer to do this and has slower growth early on in exponential phase. Furthermore, it is probably the case that cells in the Bvg- phase either elongate quicker to reach the critical size, or amount of growth, necessary to trigger cell division or are smaller in size and have a lower threshold to trigger cell

division. Biomass data points to the former scenario. In any case it is apparent that early stages of growth marked by shorter lag and higher growth rate are critical for and predictive of a high maximal yield in the Bvg- phase.

The greater consumption of glutamate per g of biomass in the Bvg+ phase suggests that more carbon dioxide is produced during growth in this phase. This would explain why more glutamate has to be metabolised, because the TCA cycle is more active in this phase and more carbon is needed to drive it, since carbon is wasted as carbon dioxide. This scenario could also explain the increased production of β-HB in the Bvg- phase, since this is thought to happen if acetyl-CoA is in excess. Since β-HB is produced in both phases, then acetyl-CoA must be in excess in both phases. CoA needs to be regenerated, and PHB is synthesised, and later β-HB. But if the TCA cycle was more active in the Bvg+ phase then more acetyl-CoA would be used to fuel the TCA cycle and there would be less available for PHB synthesis.

Bvg- phase growth is thus characterised by a shorter lag phase and faster growth early on, which means that since cells have faster doubling times then there must be something different about the way cells regulate the cell cycle in this phase. Cells in the Bvg- phase could be shorter and have lower size thresholds that must be reached before division occurs, or they could be the same size as Bvg+ cells but grow quicker to reach the thresholds that trigger division. Furthermore metabolite analysis reveals that the Bvg+ phase consumes more carbon source to produce less biomass, which is indicative of carbon being lost during metabolism, probably during the TCA cycle as carbon dioxide. If the TCA cycle is more active in the Bvg+ phase this explains the both why glutamate is consumed for less biomass and why less acetyl-CoA is available for synthesis of PHB. The reasons why the TCA cycle would be more active in the Bvg+ phase are not clear but this scenario indicates synthesis of different molecules during growth that is dependent on Bvg phase.

Chapter 4- Different genes are essential for growth of *Bordetella pertussis* dependent on Bvg phase

4.0.1 The advent of functional genomics

The advent of genome sequencing has opened up numerous avenues for functional genomic studies, by which it is meant high-throughput, genomics-based techniques aimed at gaining insight into the biology of an organism. With the availability of Illumina sequencing providing a fast and cheap way to sequence bacterial genomes, the bottleneck to understanding more about the biology of an organism has become gene function studies, i.e. it is possible by genome sequencing to know all of the possible genes of an organism, but not which are expressed or when and what their function is. The answers to these questions still require *in vitro* or *in vivo* functional studies, traditionally performed by the construction of knockout mutants, characterising a phenotype, and complementing the mutant with the functional gene. These traditional phenotypic characterisations of mutants, while fundamental, are not high-throughput, and tend to be time consuming.

To provide a resource for gene function studies, a systematic set of knockout mutants has been made in the model organism *E. coli* K12. The study was not high-throughput and was a large scale project, but nevertheless was able to provide information on gene essentiality of the organism, since 303 genes were unable to be disrupted, and designated candidate essential genes. The collection of mutants was named the Keio collection and has been made available for many gene function studies since, including the comparison of gene essentiality defined by computational prediction and experimental verification [153, 154].

4.0.2 TraDIS studies

Since then, the development of high-throughput functional studies has provided a way to look at the essentiality of all of the genes in the genome of an organism in one experiment. A technique was developed, named TraDIS (TRAnsposon Directed Insertion Sequencing), which involved the generation of an estimated 1.1 million transposon mutants of *Salmonella enterica* Typhi and the mapping of 370 000 individual insertion sites by sequencing [155]. The resolution of such a screen, involving an insertion roughly every 13 base pairs meant that every gene in the genome could be individually assayed simultaneously for essentiality, since insertions

in regions of the chromosome that are essential for growth are lethal and mutants for these genes will not be present in the mutant library. Such a screen can reveal much, including the essentiality of each gene, and due to the semi-quantitative nature of the screen, can provide information about whether loss of a gene is advantageous or disadvantageous. Furthermore, since information provided by the screen is specific to a particular growth condition, comparisons can be done to reveal information about essentiality of genes in specific growth condition versus a more general one. For example, in the study with *S. typhi*, gene essentiality was described for growth in the presence of bile, mimicking host conditions, providing information about genes that are essential for growth with and without bile [155]. Such genes are described as being conditionally essential, that is essential for growth under one condition but not another. This information is valuable in providing insight into the function of specific genes.

TraDIS has the major advantage of being high-throughput, and goes some way to bridging the gap between high-throughput genomic sequencing and lower-throughput functional studies. A similar method, Tn-seq, has been described which uses a saturated transposon library to define the relative fitness difference between mutants in different conditions [156].

Since the advent of these techniques, they have been used to predict genes involved in intrinsic resistance to aminoglycosides in *Pseudomonas aeruginosa* [157], T6SS effector proteins in *Vibrio cholerae* [158] and genes that impact sporulation in *Clostridium difficile* [159]. It must be noted that the results of a TraDIS screen are reflective of the conditions under which the experiment was carried out, and genes that are designated essential in one TraDIS experiment may not be essential under other conditions. That being said, TraDIS can be used to elucidate information about genes under conditions that mimic conditions during infection, thus helping to gain insight into genes that are responsible for survival during different stages of infection as well as changes in fitness that accompany mutations in certain genes. To this end TraDIS has been used to identify genes involved in intracellular survival of *Burkholderia pseudomallei* [160], genes involved in survival in a mouse model for *Acinetobacter baumanii* [161], and genes that were involved in infection of a mouse model for *Salmonella enterica* Typhimurium [162]. Thus TraDIS is a powerful tool for providing phenotypic data for genomes, including new candidate genes involved in antibiotic resistance, virulence, and growth and survival.

4.0.3 Overview of TraDIS methodology

The Tn-seq method uses a Himar I *Mariner* transposon, with recognition sites for the type II restriction endonuclease MmeI incorporated. This enzyme makes a 2 base pair staggered cut 20 base pairs downstream from the recognition site. When DNA from a transposon mutant library is cut the fragments contain the transposon ends plus 16 base pairs of flanking genomic DNA, which through DNA sequencing can be used to determine the location of the transposon. Furthermore the 2bp overhangs facilitate the ligation of an adapter. High-throughput sequencing is used to determine the flanking 16bp flanking sequence, and thus the location in the genome in which the transposon insertion occurred. TraDIS is a very similar method, but any transposon can be used, and without the restriction site, giving the advantage to being active in a range of species. Generally the Tn5 transposon is used. The procedure contains more steps than Tn-seq, including a shearing step [163, 164].

The number of sequence reads corresponding to a particular insertion is proportional to the frequency of that insertion mutant in the pool. Insertion index can be calculated by normalising the number of insertions for a given gene by the gene length (since the longer the gene the more insertions would be expected). If the distribution of the insertion indices is plotted a bimodal distribution is obtained where essential genes have an insertion index of 0. Likelihood ratios (LR) can be calculated based on the distribution and essential genes can be identified from these by setting cut-offs [155, 164]. Typically, a gene with a $\log_2$-LR of less than -2 (meaning that a gene is four times more likely to be essential than nonessential) is designated essential.

With respect to comparing differences between two different libraries exposed to different conditions, there are three potential outcomes that could occur with respect to a particular gene following the selection event of the mutant library. The first is that the frequency with which a particular mutant appears in each library is the same, which would indicate that the gene is neutral with regards to fitness and that knocking out the gene does not have an effect on fitness. The second is that the frequency with which the mutant is obtained is decreased in one condition over the other, indicating that the gene is advantageous in this condition. The final possible outcome is that the mutant does not appear in the population of mutants in one of the conditions indicating that the gene is essential for growth under this condition but not the other and the gene is a conditionally essential gene. In practice a large range of

fitness changes are seen with regard to all genes in a TraDIS experiment, but high-throughput sequencing is sensitive enough to detect changes in fitness that affect the growth rate by as little as 5% [163].

4.1 Results

4.1.1 TraDIS reveals information about biology of *B. pertussis*

TraDIS was performed for BP536 growth on charcoal agar under both Bvg+ and Bvg- phase conditions, recovering transposon mutants in both Bvg phases. Mutants from each phase were pooled and genomic DNA extracted and sequenced to find the location of the transposon.

300,581 and 316,281 unique insertion sites were identified in the Bvg+ and Bvg- phases respectively, corresponding to an average of one insertion every 13.6 and 12.9 bp across the genome of *B. pertussis*. This provides a very high-resolution transposon library. Calculating the gene insertion index makes comparison between genes possible and is done by normalising the number of insertion sites in a gene for the gene length. When the gene insertion indices are plotted the distribution is bimodal, with essential genes at 0. Genes that had a log2-likelihood ratio (log2-LR) of less than -2 were taken to be essential, thus genes with a log2-LR greater than -2 were designated non-essential.

Performing TraDIS in two different conditions, Bvg+ and Bvg- conditions, provides information about genes that are essential for growth in both Bvg conditions and therefore genes that are only essential for growth in one Bvg phase, called conditionally essential. Ambiguous genes are described, which are genes that cannot with confidence be called essential or non-essential. Finally, fitness affected genes are described, which are genes that have a significantly greater fitness cost between Bvg phases (i.e. the frequency at which a mutant is seen is different between the two phases).

396 genes have been designated essential for growth on charcoal agar at 37°C. A further 19 genes are also essential but only for Bvg+ phase growth, while a further 79 gene are essential only for Bvg- phase growth. These genes are referred to as conditionally essential because they are essential for growth based on certain conditions, in this case activity of the Bvg two-component system.

Many of the essential genes are involved in major cellular processes. For example, of the eight genes making up the DNA polymerase III holoenzyme, all but two are essential. *BP2022* is not essential, and *dnaQ*, coding for the epsilon chain, is essential only in the Bvg- phase. Genes for 19 aminoacyl-tRNA synthetases were also designated essential (a gene for asparaginyl-tRNA synthetase was unable to be identified). Genes involved in DNA maintenance or repair are also designated as essential and include *gyrA* and *gyrB*, *ligA*, and *parC*, though *parE* is only essential in the Bvg- phase. Genes in areas of interest, such as metabolism and peptidoglycan biosynthesis, which are pertinent to this study, are discussed.

4.1.2 Bvg: Conditionally essential genes

TraDIS was performed with selection in the Bvg+ and Bvg- phases. A total of 396 genes are essential for growth of BP536 regardless of Bvg phase. A further 19 genes are essential for growth only in the Bvg+ phase and a further 79 genes are essential for growth only in the Bvg- phase. These genes are called conditionally essential because they are essential only in one condition or another.

The 19 genes that are conditionally essential for growth in the Bvg+ phase include genes involved in cell division and DNA repair. ParA is a chromosome partitioning protein, while *ruvA, ruvB* and *ruvC* are involved in resolving Holliday junctions. Other genes essential only in the Bvg+ phase are the uridylyltransferase *glnD*, which is involved in sensing the nitrogen status of the cell, and *ribH*, which is involved in riboflavin metabolism. The sigma factor *sigE* is also essential only in the Bvg+ phase, as was the *tex* gene involved in regulation of toxins, previously shown to be essential [165]. The *tex* gene was so called because of its perceived function in toxin expression and was speculated to have a role in transcription. Of the 19 genes that are essential only in the Bvg+ phase, 9 were unable to be assigned a function based on amino acid homology to other proteins. Thus the existence of genes that are conditionally essential for growth dependent on Bvg phase reveals differences in physiology between the two Bvg phases. Conditionally essential genes are displayed in table 5.

Function	**Gene**
Nitrogen metabolism (sensing)	*glnD*
Cell division	*parA*
Riboflavin metabolism	***ribH***
DNA repair	*ruvA*, ***ruvB***, *ruvC*
Transcription	*sigE, tex*
Translation	***infA***, ***rnpA***
Unknown	*BP0183, BP0184,* ***BP0953****, BP1296, BP2197,* ***BP3016****, BP3148, BP3151, BP3819*

Function	**Gene**
Amino acid biosynthesis	*argC, aroA, aroC, panD,* ***hisA****, hisB, hisH, hisI*
Intermediary metabolism	***odhL***
LPS biosynthesis	*bplF*
Cell wall synthesis	*dadX, mrdA, mrdB, mreB, mreC, mreD*
Sugars metabolism	*pgm*
DNA replication	*dnaQ,* ***parE***, ***recG***
DNA repair	*ung, dksA*
Cell division	***ftsY***, ***minE***

Translation	*ksgA, rbfA,* ***rplK****,* ***rpmB****, rpmF,* ***rpmH****,* ***rpsL****, efp,* ***rmsH*** *(mraW BP3030), BP2689*
Lipoate metabolism	*lipA, lipB*
Energy metabolism	***nuoA****, rubA, sdhA, sdhB, sdhC, sdhD, BP2359*
Transport	***ompA****, secE,* ***BP2338***
Carbohydrate metabolism	*rfbB, BP0693, BP1500, BP3403*
Transcription	*rpoZ, BP0991, BP1814*
Chemotaxis	*tsr*
Protein modification	***lgt,*** *ptpA*
Unknown	***BP0035****, BP0102, BP0163, BP0240,* ***BP0247****, BP0706, BP1127, BP1245, BP1413, BP1721, BP1837, BP1903, BP1907, BP2011, BP2489, BP2799, BP2956, BP3127, BP3128, BP3341,* ***BP3345****, BP3390, BP3488*

Table 5- Genes that are conditionally essential for growth on charcoal agar in the Bvg+ phase (top) and Bvg- phase (bottom). Function refers to a broad pathway that these genes are thought to be involved in. Genes in bold refer to genes also designated as ambiguous in the other phase meaning that these genes, while designated conditionally essential based on TraDIS data may actually be essential in both phases.

Genes that are conditionally essential for growth are presented in table 5. One of the most striking things about the genes conditionally essential only in the Bvg- phase is that an entire operon, *mreBCDAmrdAB*, containing genes involved in cell shape and wall maintenance is essential only in this phase. It has been shown previously that some of these genes are essential for growth in *E. coli*, under normal conditions but that mutants can grow in certain conditions, for example during slower growth in minimal media [166].

There are a number of metabolic genes that are essential for growth only in the Bvg- phase including *argC, panD, aroA, aroC, and odhL*. *argC* catalyses the third step in the synthesis of arginine from glutamate. It is not clear how this gene, performing one-step in a linear pathway can be differentially essential while other genes are not, although there may be redundancy amongst some of the steps, for example *argJ* and *argA* catalyse the same reaction. *panD* catalyses the conversion of L-aspartate to β-alanine, in the pathway of CoA biosynthesis. All other genes involved in other steps of this pathway are essential in both Bvg phases. *aroA* and *aroC* are involved in subsequent steps in the formation of chorismate, a precursor in the synthesis of phenylalanine, tryptophan and tyrosine. Most other genes involved in this pathway are non-essential in both Bvg phases, although *aroQ* is essential in both phases, and *pheA*, which catalyses the first step in synthesising phenylalanine from chorismate has significantly less insertions in the Bvg- phase. The reasons why different parts of a pathway are differentially essential are not clear, although it suggests that there are alternative ways of forming these precursors, or alternative ways of synthesising or obtaining these amino acids.

Another metabolic gene, *odhL* forms a component of the 2-oxoglutarate dehydrogenase involved in the TCA cycle, specifically forming NADH from NAD^+ during this reaction. This may point to more of a reliance on maintaining NADH levels in the Bvg- phase.

Of the 19 genes that are essential only in the Bvg+ phase 6 are ambiguous in the Bvg- phase, and of the 79 that are essential only in the Bvg- phase 18 are ambiguous in the Bvg+ phase. This provides a caveat with the TraDIS method whereby a small number of genes with a very small number of insertions aren't strictly identified as essential, but for which in laboratory settings it may be extremely difficult to isolate a mutant. Some of these genes that are essential in one phase and are ambiguous in the other (identified by bold typeface in table 5) could be essential in both phases and not differentially essential as suggested. That said there are still a number of genes that are truly differentially essential in both phases.

4.1.3 Electron Transport Chain

B. pertussis has a well-defined metabolism. It is not able to grow anaerobically and uses oxygen as sole terminal electron acceptor [167]. The electron transport chain

generates a proton motive force, which is used to generate ATP, and thus it would be expected that components of this process are essential for cell growth. The predicted electron transport chain is presented in figure 19.

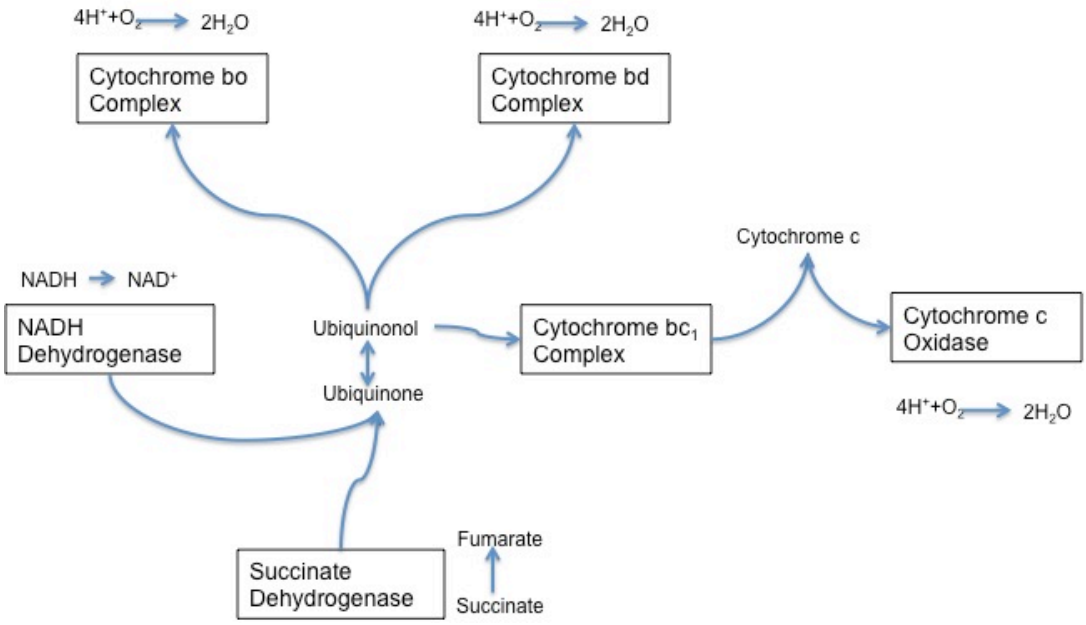


Figure 19– The predicted electron transport chain of *B. pertussis*. Movement of electrons is shown by curly arrows. Briefly, the NADH dehydrogenase transfers an electron from NADH to ubiquinone, causing its reduction to ubiquinol. Succinate dehydrogenase also reduces ubiquinone, but with an electron liberated during the oxidation of succinate to fumarate. Ubiquinol can be oxidised back to ubiquinone, transferring an electron to cytochrome c by the cytochrome bc_1 complex. Cytochrome c is then oxidised by the cytochrome c oxidase, reducing oxygen to water as the terminal electron acceptor. Alternatively, the cytochrome bo complex or the cytochrome bd complex can both couple the oxidation of ubiquinol to the reduction of water directly [168].

The electron transport chain provides protons to the PMF for use by the ATP synthase in the synthesis of ATP. It is no surprise to find that seven genes that code for the ATP synthase are essential. What is perhaps surprising though is that *atpC*, coding for the epsilon chain of the enzyme complex, is not essential.

Similarly, of the fourteen genes coding for NADH dehydrogenase, the first complex in the electron transport chain, thirteen are essential, with *nuoA*, coding for chain 7, being non-essential. These are the only parts of the electron transport chain that are essential. The four genes coding for succinate dehydrogenase, *sdhABCD*, the only enzyme to take part in both the electron transport chain and the TCA cycle, are essential only in the Bvg- phase. The genes coding for cytochrome c reductase,

petABC, are non-essential but mutants are more fitness affected in the Bvg- phase, defined as there being statistically less reads recovered from the Bvg- phase than Bvg+ for this gene. Furthermore genes coding for cytochrome c oxidase are non-essential for growth, as are the genes coding for the cytochrome bd complex and the cytochrome bo complex.

4.1.4 Glutamate metabolism

Glutamate as sole carbon source can support growth [115] and is the basis for growth in liquid Stainer-Schotle broth [117]. Thus *B. pertussis* must be able to synthesis all cell components from glutamate, and it stands to reason that glutamate metabolism must be at the centre of *B. pertussis* metabolism.

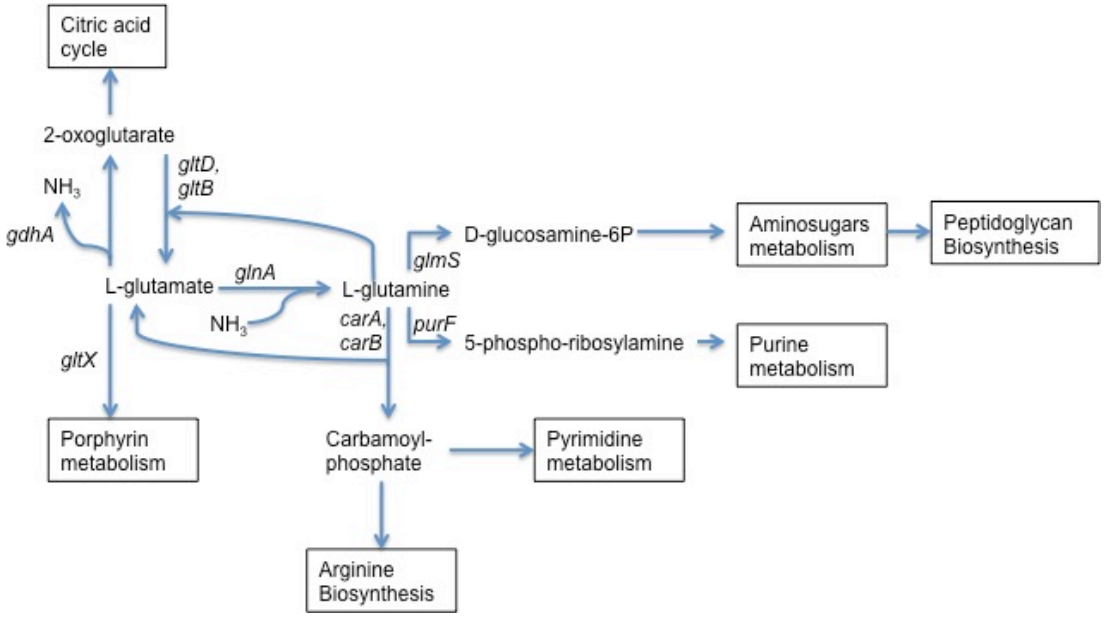


Figure 20– Metabolism of glutamate in *B. pertussis*. The various fates of glutamate in *B. pertussis* are shown including how pathways feed into the citric acid (TCA) cycle and amino sugars metabolism. Genes responsible for reactions are shown in italics.

Glutamate metabolic pathways are shown in figure 20. There are four genes involved in glutamate metabolism that are essential for growth on charcoal agar, *glnA*, *glmS*, *carA* and *carB*. *glnA* metabolises L-glutamate to L-glutamine, while *glmS* metabolises glutamine to D-glucosamine-6-P which is a precursor of aminosugars. *carA* and *carB* metabolise L-glutamine to carbomoyl-phosphate and this is used to feed into pyrimidine and arginine synthesis pathways. It might have been expected that more

genes in this immediate pathway were essential, though the conditions of growth in which the TraDIS experiment was undertaken was not with glutamate as the sole carbon source as it was not possible to obtain single colonies on a defined agar medium. Charcoal agar is a complex medium and it is likely that in this condition *B. pertussis* metabolises a variety of sources of carbon. However, glutamate may still be a main source of carbon and it is still reasonable to suggest that glutamate metabolic pathways would have evolved to be at the centre of metabolism in *B. pertussis*, since the bacterium is able to make all components of the cell from glutamate, and other carbon sources alone support little or no growth. There may also be redundancy covering parts of this pathway, for example BP536 has two versions of *gdhA*, meaning that a mutation in one gene wouldn't necessarily lead to a loss of viability.

4.1.5 TCA cycle

The TCA cycle is a central part of metabolism. From here intermediates are made that contribute to the synthesis of amino acids, aminosugars for making the cell wall and is a source of reducing power for feeding into the electron transport chain. It was assumed for many years that *B. pertussis* did not have a fully functional TCA cycle, but that was later disproved. Not only was it shown that all of the genes were present, but that the genes thought to be missing were expressed and produced functional enzymes [123].

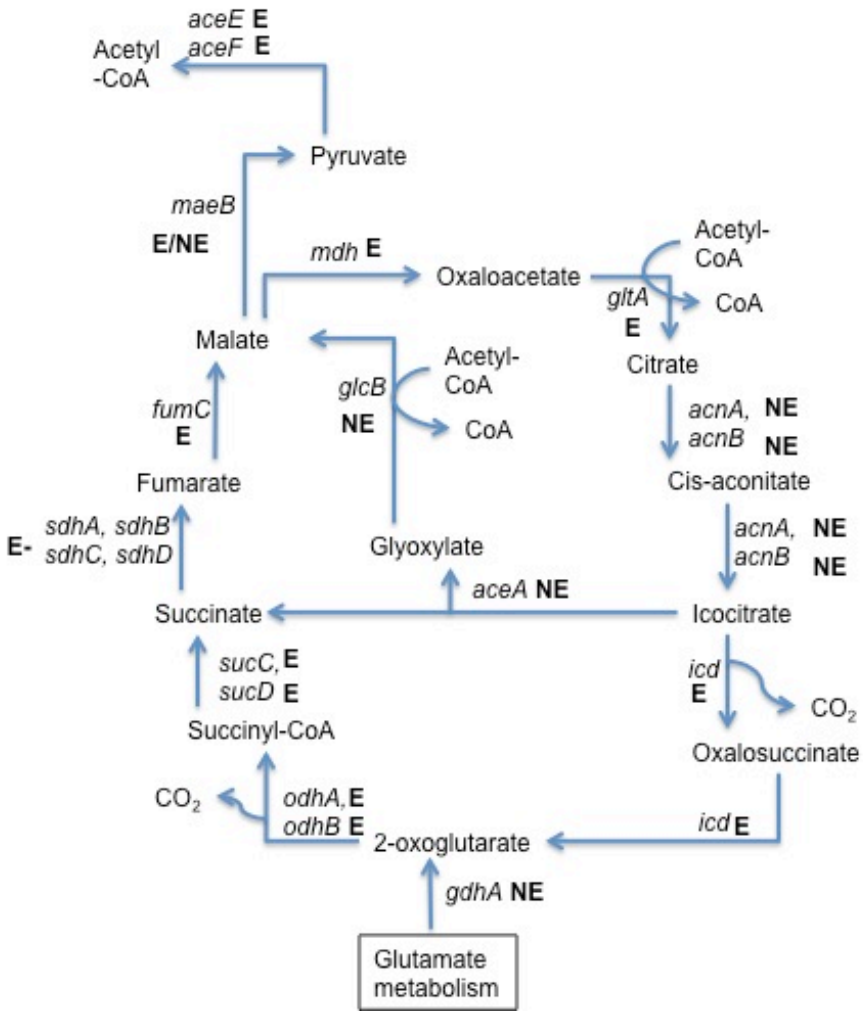


Figure 21– The essentiality of the TCA cycle of *B. pertussis*. Genes are labelled either E to denote that gene is essential, or NE to denote that gene is non-essential. E- denotes that genes *sdhA-D* are essential only in the Bvg- phase.

The majority of genes coding for enzymes of the TCA cycle are essential in *B. pertussis*, including *mdh, fumC, sucCD, odhAB, icd, gltA, aceEF*. The genes coding for succinate dehydrogenase (*sdhABCD*) are essential only in the Bvg- phase. The few nonessential genes include the genes coding for aconitate hydratase (*acnAB*) and the glyoxylate bypass (*aceA* and *glcB*).

The *B. pertussis* genome contains two genes annotated as *aceE*, *BP0993* and *BP1121,* these genes both code for the same subunit of pyruvate dehydrogenase, and while the former is essential, while the latter is not. The protein sequences contain 55% amino acid identity to one another, which may indicate that the essential gene, *BP0993*, has optimal activity and the bacteria cannot tolerate losing it, while the loss of *BP1121* can be tolerated because it has suboptimal activity and the cells can

continue with only *BP0993*. The genome of *B. pertussis* contains three genes annotated *maeB*. Two, *BP1064* and *BP3456,* are essential while one, *BP1120,* is non-essential. It's not clear why two out of three genes that apparently perform the same function are essential, although *BP1120* shares only 64% identity at amino acid level with the other two genes, which share 97% identity with each other, so it could be functionally different.

4.1.6 Peptidoglycan synthesis

Peptidoglycan is a key component of the cell wall of bacteria, and gives cells shape and rigidity. It would be expected that enzymes involved in making peptidoglycan are essential for growth, since preventing synthesis of the cell wall, with use of some antibiotics for example, generally leads to cell death.

The majority of the genes of *B. pertussis* that are annotated as functioning as part of the peptidoglycan synthesis pathway are essential, including *murABCDE, ddlB, murX, muG, BP2771* (a flipase), and *ftsI*. Of these genes only *ftsI* is a PBP, which catalyse essential crosslinking reactions during peptidoglycan synthesis and are the targets of the β-lactam class of antibiotics, although there are up to 9 PBPs coded for in the *B. pertussis* genome, which could provide redundancy. A further two genes are essential only in the Bvg- phase, *mrdA* and *BP0102*, both of which are PBPs (PBP2 and PBP6 respectively). Another PBP, *BP3655* (PBP1A) is more fitness affected in the Bvg+ phase than in the Bvg-. 4 PBPs are nonessential in both Bvg phases; *BP0905, BP2754, BP0326* and *BP1051*.

Although bacteria code for many PBPs, they play slightly different roles within the cell. The differing degrees of essentiality of PBPs dependent on Bvg phase may reflect the different biological requirements that cells have in the different Bvg phases. PBP2 is key for peptidoglycan synthesis for elongation of the cell [169, 170] and it may be that this is a more important process for cells in the Bvg- phase than in the Bvg+ phase. Alternatively these data may suggest that the peptidoglycan synthesised in the two Bvg phases is chemically different and therefore tolerates the loss of specific PBPs differently.

4.1.7 Fitness affected

Mutations of some non-essential genes will lead to a fitness cost. TraDIS is a quantitative tool, since it provides a measure of how many insertions were recovered for each gene and differences between two libraries selected in different conditions. Fitness affected genes are defined as genes that have a significantly different ($p<0.05$) number of insertions dependent on Bvg+ and Bvg- phase growth. This means that mutants are significantly less fit in one phase than another. Overall there are 50 genes more affected for fitness in the Bvg+ phase and 51 genes more affected for fitness in the Bvg- phase. These are depicted in table 6.

Function	**Gene**
Redox homeostasis	*trxA, trxC, mrsB*
Protein folding	*ppiD*
Capsule biosynthesis	*tviD, BP1619, BP1620, kpsM, kpsT, kpsE, wza*
Folate metabolism	*folD*
Ion transport	*BP2806*
Cell division	*ftsB, BP3563,* ***BP3822***
Amino acid biosynthesis	*soxB*
Nitrogen metabolism (sensing)	***glnD***
DNA replication	*dps, bpH1*
Transcription	*BP0667,* ***tex****, bvgA*
Translation	*BP1244*

Unknown	*BP0062, BP0063, BP0063A, BP0065, BP0066, BP0076,* ***BP0184****,* ***BP1296****, BP1426, BP1569, BP1769,* ***BP2197****, BP2438, BP2519, BP2523, BP2846, pcp, BP3143, BP3145,* ***BP3151****, BP3277, BP3402, BP3467, BP3561, BP3562,* ***BP3819***

Function	**Gene**
Amino acid biosynthesis	*pheA, metE*
Phytoene synthesis	*BP1219*
Folate metabolism	*BP3066*
Cell wall synthesis	*ampG,* ***BP0102****,* ***mreB****,* ***mreC****,* ***mreD****,* ***mrdA****,* ***mrdB****,* ***dadX****, BP3214, BP3268*
Sugars metabolism	*rfbC*
Nucleotide metabolism	*dut, pyrF*
DNA repair	***ung***
Cell division	*ftsE, BP3833*
Translation	*typA,* ***ksgA***
Energy metabolism	*petA, petB, petC*
Ion transport	*corC*
Amino acid transport	*BP3428*
Transcription	***BP1814****, BP2308, metR, BP2720, BP3865*
Unknown	*BP0205, BP0608, BP0690, BP0900, BP0952, BP1061,* ***BP1127****,* ***BP1413****, BP1582,* ***BP1903****, BP2330, BP2770,* ***BP2956****,* ***BP3128****, BP3347,* ***BP3488****, BP3752, BP3762, BP3867*

Table 6– Fitness affected genes in the Bvg+ (top) and Bvg- phase (bottom). Genes in bold are also essential in that phase.

In the Bvg+ phase a mutant of *bvgA*, the response regulator of the Bvg system Is fitness effected in the Bvg+ phase. This means that this mutant of *bvgA* is less fit in the Bvg+ phase than in the Bvg-. In the Bvg- phase BvgS is not signalling to BvgA,

so it would be expected that a mutant of *bvgA* would behave like other strains in this condition. However, in the Bvg+ phase BvgS is active and phosphorylating BvgA, and the other mutants on the plate would be producing virulence factors, while a *bvgA* mutant would not be. The Bvg regulon is large and BvgA is required for the proper expression of many genes, so it is easy to see that a mutant of this transcription factor would be affected for fitness. Furthermore, transcription of the *bvgA* gene is repressed in the Bvg- phase so it might be expected that a *bvgA* mutant would be less fit in the Bvg+ phase than in the Bvg-, since it is in this phase that it is more highly expressed.

There are mutants of genes involved in the synthesis of the capsule that are fitness affected in the Bvg+ phase, which is surprising as these genes are known to be maximally expressed in the Bvg- phase [171]. It has been shown previously that mutations in capsule synthesis genes, specifically *kpsT* and *kpsM*, affect expression of virulence genes, possibly through an indirect interaction with BvgS [172]. This observation, together with the one that mutants of *bvgA* are fitness affected in the Bvg+ phase, suggest that impairing expression of virulence genes in the Bvg+ phase affects fitness when in competition with other mutants.

Different genes involved in cell division are fitness affected, suggesting different ways of regulating cell division dependent on Bvg phase, while mutants of genes involved in metabolism are also fitness affected. *soxB,* involved in the conversion of sarcosine to glycine and mutants are fitness affected in the Bvg+ phase, while *metE* and *pheA* are involved in methionine and phenylalanine biosynthesis respectively and mutants are fitness affected in the Bvg- phase. The genes *petABC,* coding for the cytochrome bc_1 complex, are fitness affected in the Bvg- phase, providing evidence for a difference in the way that the electron transport chain operates depending on Bvg phase, possibly the Bvg- phase catalyses oxidation of ubiquinol via this cytochrome complex over others. The observation of different metabolic genes with different degrees of essentiality with respect to Bvg phase is interesting and may reflect the different requirements that growth in each phase has. More metabolic genes are essential or fitness affected in the Bvg- phase, perhaps reflecting greater constrains of Bvg- phase growth or a more varied metabolism.

4.1.8 *mreB* is conditionally essential for growth on solid media

Transposon-directed Insertion Sequencing (TraDIS) was used to identify genes essential for growth of *B. pertussis* in both the Bvg+ and the Bvg- phases under standard lab conditions on charcoal agar. The data showed that the *mreB* gene was essential for growth in the Bvg- phase but not in the Bvg+ phase. An *mreB* mutant was made and used to validate the result from the TraDIS that this mutant is conditionally viable depending on Bvg phase. An *mreB* mutant was constructed. Briefly, PCR was performed using the primers listed in Chapter 2 obtain the two fragments *mreB* right flanking region, *mreB* left flanking region. The pCR8*mreB* vector constructed by golden gate contained an insert with roughly 500bp of the sequence upstream and 500bp downstream of the *mreB* gene either side of a kanamycin cassette. A Gateway reaction was performed to recombine this insert into pss4940, and a conjugation and selection was performed as described above. Selection on kanamycin ensures that the second recombination event preferentially occurs to recombine the wild-type fragment onto the chromosome along with the kanamycin cassette. Clones were analysed by PCR for presence of the knockout construct and absence of the wild-type *mreB* gene. The viability of an *mreB* mutant under Bvg+ and Bvg- phases was tested. Data is shown in figures 22 and 23.

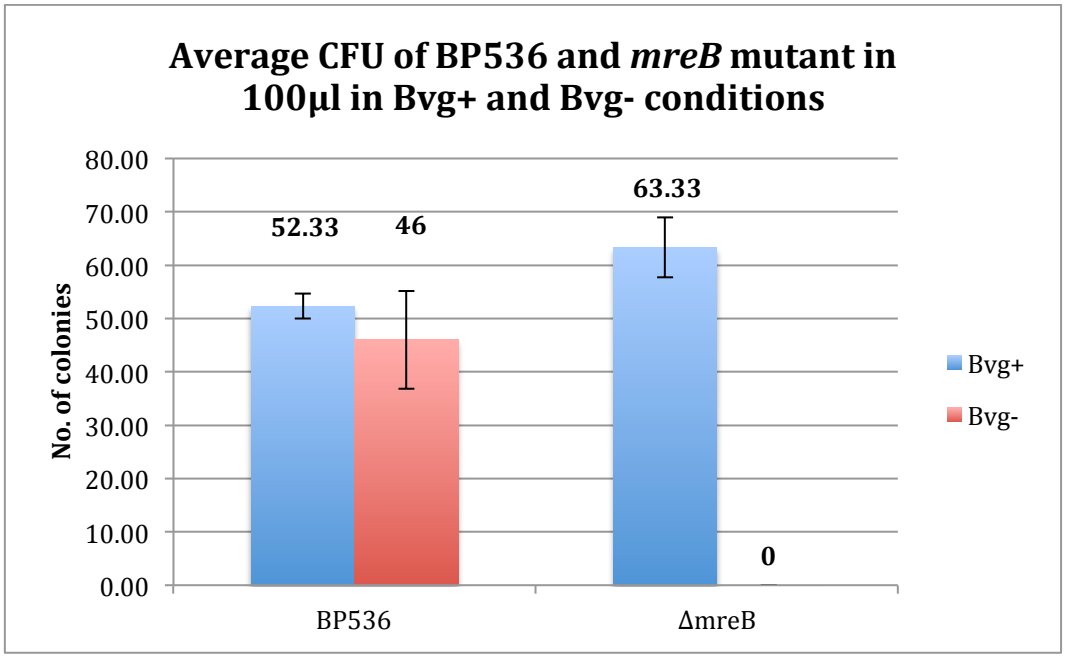


Figure 22- Viability of the *mreB* mutant in Bvg+ and Bvg- conditions. BP536 had a similar CFU count after 7 days regardless of Bvg phase. The *mreB* mutant had a similar viability to BP536 in the Bvg+ phase but no colonies were recovered when Bvg- phase was induced. Data is an average of three technical replicates; error shows the standard error of the mean.

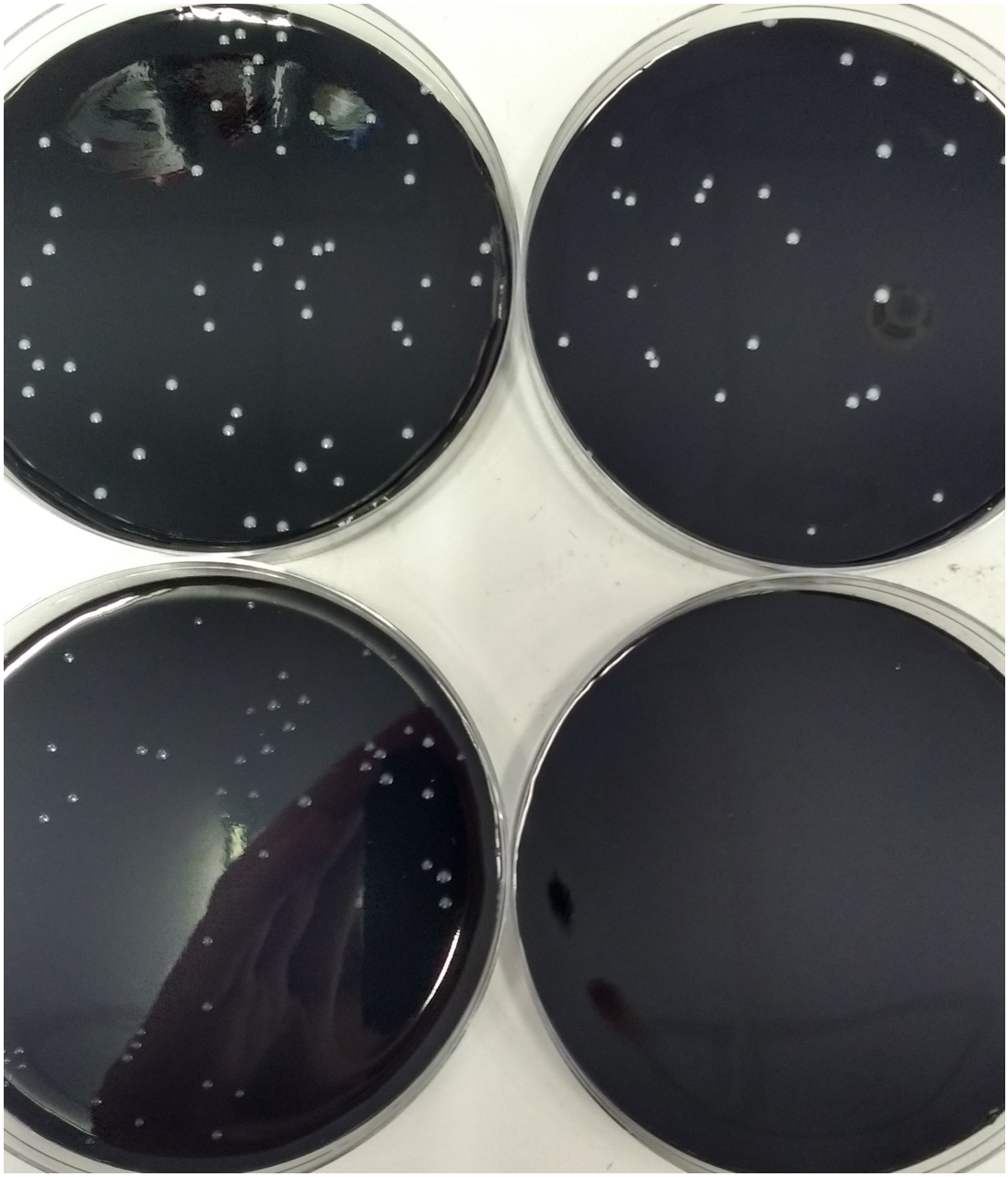


Figure 23- Photos of (clockwise from top left) charcoal agar with BP536 Bvg+, BP536 Bvg-, *mreB* mutant Bvg-, *mreB* mutant Bvg+. Similar numbers of bacterial colonies grew in all cases except for the *mreB* mutant on plates supplemented with 50mM magnesium sulphate, which showed no growth.

There was no difference between the number of BP536 colonies recovered under Bvg+ and Bvg- conditions. Furthermore the number and size of colonies recovered for the mutant on charcoal agar in Bvg+ conditions was not different from the wild-type demonstrating that *mreB* is not required for Bvg+ phase growth on charcoal agar.

When the mutant was plated onto charcoal agar containing magnesium sulphate to induce the Bvg- phase no colonies were recovered demonstrating that the

mreB gene is essential for growth of BP536 on charcoal agar in the Bvg- phase and validating the observation made by the TraDIS data.

This experiment was repeated with Bordet-Gengou agar containing 10% defibrinated horse blood and the same results were observed demonstrating that *mreB* is essential for growth of BP536 on these two agars in the Bvg- phase.

4.1.9 Preliminary growth of the *mreB* mutant

To investigate if *mreB* is essential for growth in liquid broth, the mutant and BP536 were grown in SS broth but only weak growth was observed for the mutant. The observation was made that the *mreB* mutant was viable in SS broth, but growth was dependent on inclusion of heptakis in the medium (figure 24).

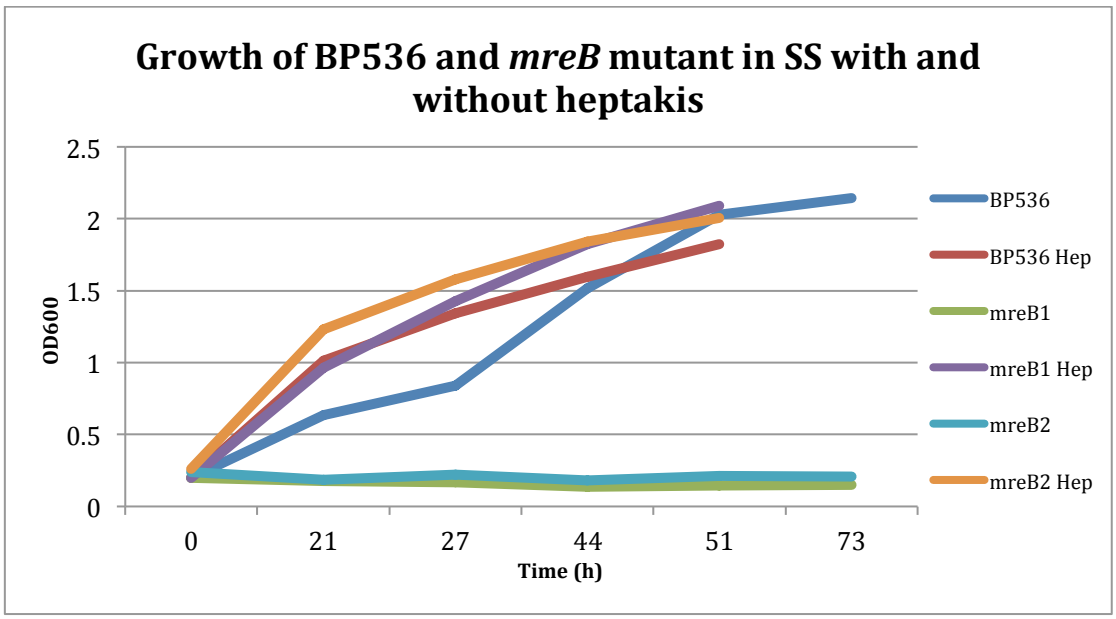


Figure 24– Growth of BP536 and two different clones of the *mreB* mutant (mreB1 and mreB2) in SS broth with and without heptakis. Strains were grown in conical centrifuge tubes with samples for measurements of optical density were taken over time. Two different media were used, SS medium with and without heptakis. "Hep" denotes the cultures to which heptakis was included while the other cultures were grown in SS medium without heptakis.

Addition of heptakis enhanced the growth of BP536, causing it to grow faster in early stages of growth, but where growth was obtained for the *mreB* mutant cultures there was no growth defect seen relative to BP536.

4.1.10 *mreB* is not required for growth in SS broth

A growth assay was performed in a 96-well plate in SS broth containing 1g/l heptakis and growth was observed. When an OD_{600} of 1.0 was reached 50µl was removed from each culture, half of the cultures had 50µl of fresh culture added and half had 50µl of fresh culture added which contained magnesium sulphate to modulate to Bvg- conditions. Growth of the cultures is displayed in figure 25.

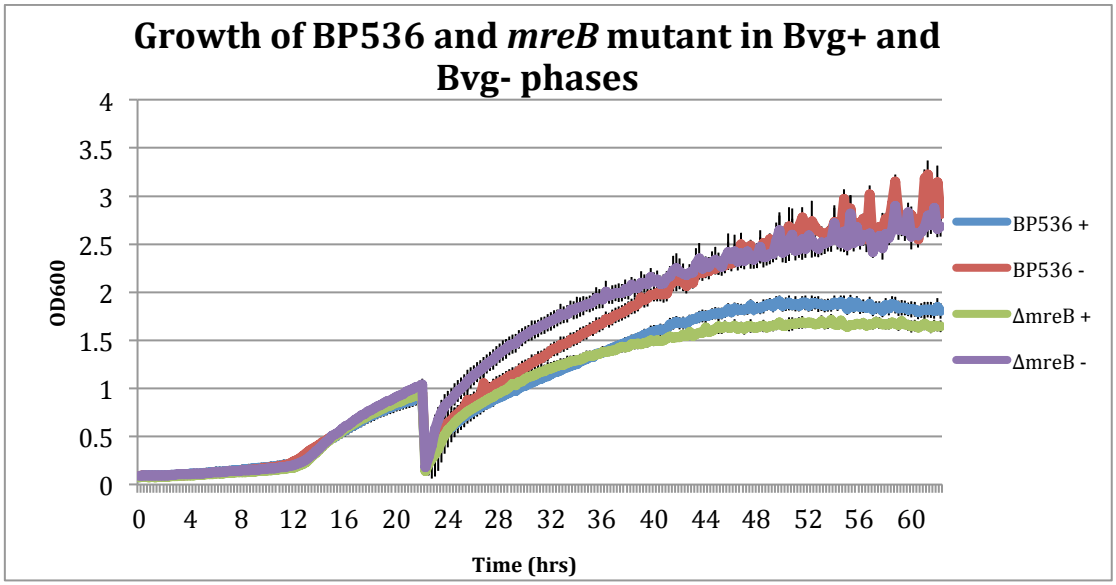


Figure 25– Growth of wild-type BP536 and *mreB* knockout mutant in SS broth containing heptakis. +: cultures grown under Bvg+ conditions –: cultures grown under Bvg- conditions. Growth shown is an average of ten cultures in the same 96-well plate. Error bars show standard deviation.

Growth of all of the cultures was very similar until modulation was induced, revealing that *mreB* is not required for growth in SS broth and loss of *mreB* does not give an observable growth defect, at least during early growth.

Following modulation, all of the cultures continued to grow but Bvg- cultures grew to a higher cell density. This happened for both BP536 and *mreB* mutant Bvg- cultures.

Therefore the *mreB* gene was not required for growth in SS broth with heptakis, although growth was poor when heptakis was absent from the medium. Growth of the mutant was unaffected by loss of the gene in either Bvg+ or Bvg- phase when compared to BP536. The observation from TraDIS that *mreB* is essential for growth in the Bvg- phase is true for the solid media tested but not liquid medium. This is similar to an observation reported previously where mutations of *mre* in

Salmonella typhimurium were lethal on solid media but not liquid [173]. The difference, however, in *B. pertussis* is that the *mreB* mutant is viable when grown on solid media in the Bvg+. In this condition growth is purely conditional depending on activity of the Bvg two-component system and not type of medium used or a compensatory mutation. To my knowledge this is the first instance in which growth of an *mreB* mutant is dependent on activity of a two-component system.

4.1.11 *mreB* is required for growth of rod-shaped cells

mreB was previously referred to as a rod-determining gene as loss of this gene resulted in loss of the rod shape and growth of spherical cells which would die. MreB is now thought of as a bacterial homologue of actin with varying roles in the cell including directing cell synthesis along the axes and providing some spatial partitioning to the cell [174].

BP536 and the *mreB* mutant were grown on charcoal agar and imaged by TEM and a shape difference was observed (figure 26).

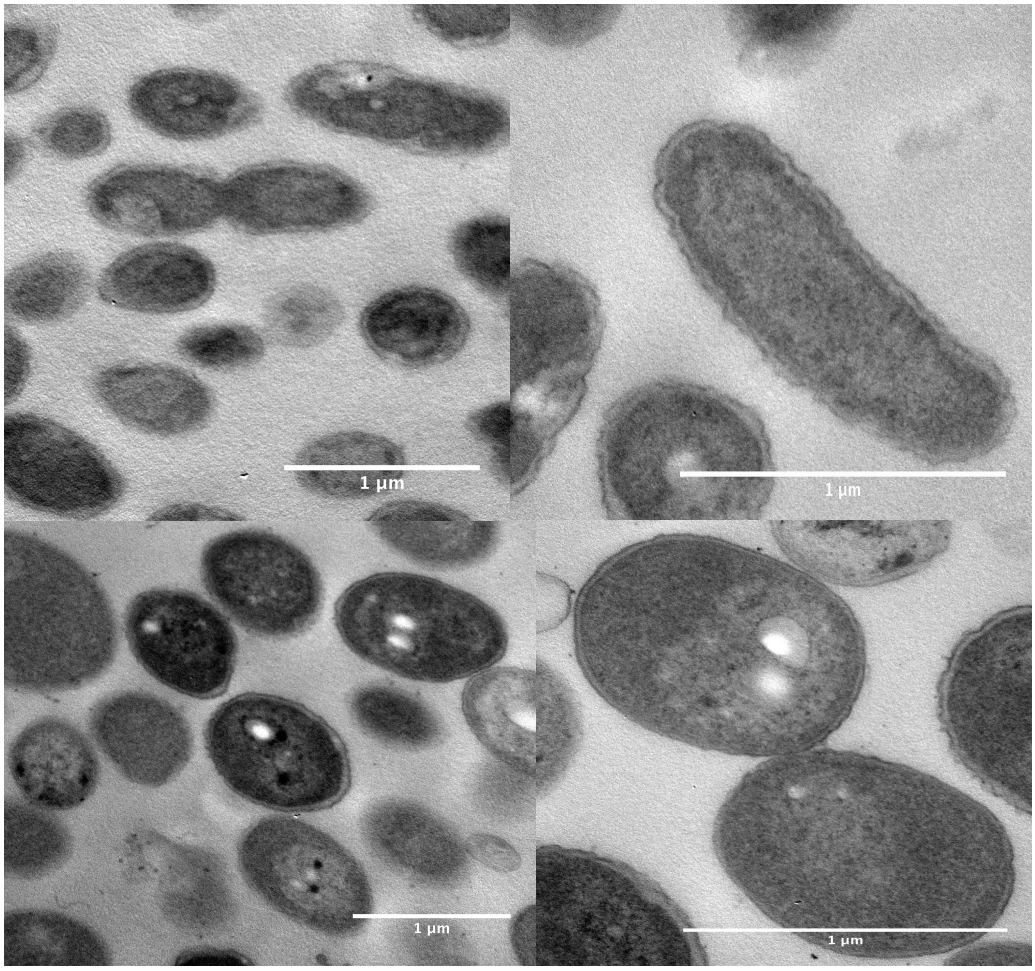


Figure 26– BP536 (top) and *mreB* mutant (bottom) imaged by TEM. All scale bars are 1µm. BP536 cells are rod-shaped, while the *mreB* mutant cells are spherical. There are more white spots observed in the cells of the *mreB* whilst mutant samples frequently showed more cell debris than BP536.

BP536 cells were rod-shaped and small, while the mutant cells were larger and spherical. The mutant cells were less uniform in size, and had a less defined cell envelope. More of what may be lipids inside the cells were evident in the mutant as well as cell debris, which is indicative of unhealthy cells. Measurements of the length and width of cells is shown in figure 27.

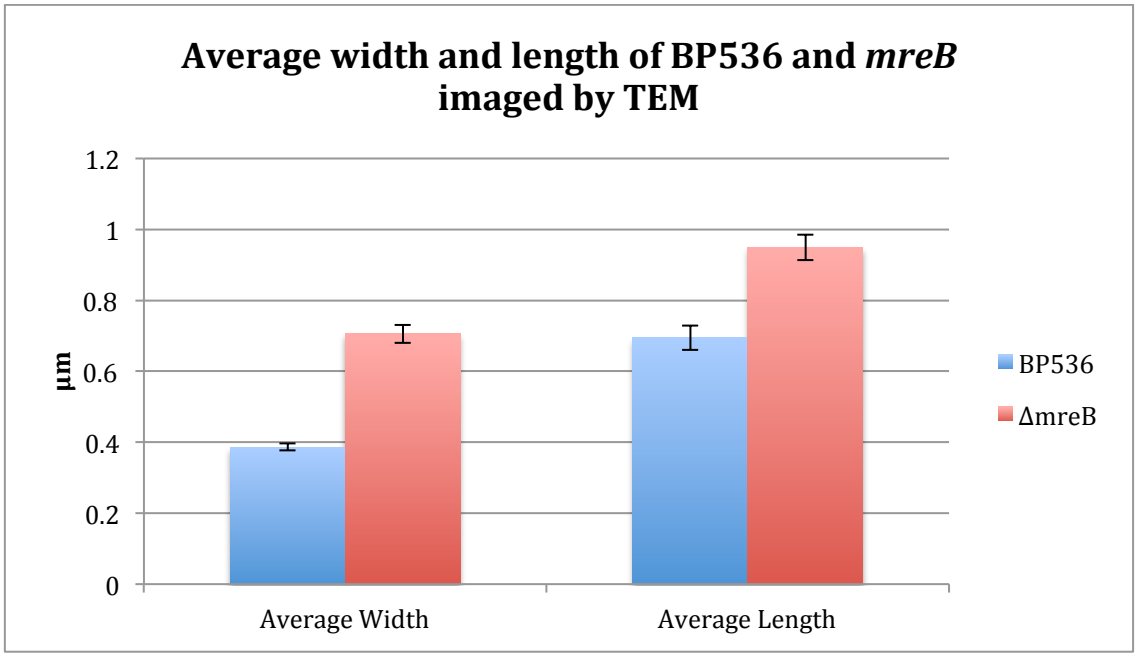


Figure 27- Average widths and lengths of BP536 and *mreB* mutant imaged by TEM. Cells were chosen at random and measured using ImageJ. Error shown is standard error of the mean. n=56 (BP536), n=20 (*mreB* mutant)

The average width of BP536 cells was 0.387µm, while the width of *mreB* mutant cells was 0.706µm, which was significantly different ($p=4.212x10^{-12}$). The average length of BP536 cells was 0.694µm and the average length of *mreB* mutant cells was 0.950µm, this was also significantly different ($p=2.470x10^{-6}$).

The average volume of the cells was also measured, approximating the volume by measuring it as that of a cylinder (shown in figure 28).

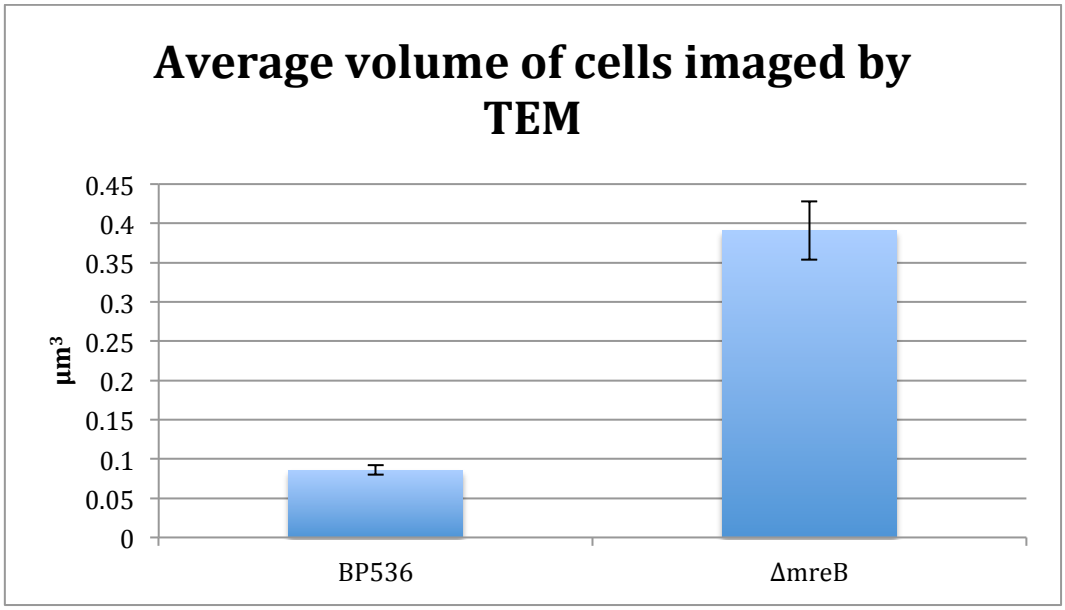


Figure 28- Average volumes of BP536 and *mreB* mutant imaged by TEM. Data was calculated on the assumption that the cells are cylindrical using the formula $v=\pi r^2 h$ where r is the radius (taken to be half the width of the cell) and h is the height (taken to be the length). Error shown is standard deviation of the mean. n=56 (BP536), n=20 (*mreB* mutant)

The average volume of BP536 cells was 0.0858µm^3 while the average volume of the *mreB* mutant cells was 0.391µm^3, which was significantly larger (p=0.00345).

Therefore without MreB cells grow bigger in both dimensions (width and length), which means cells also have a larger volume than BP536 cells. This in in agreement with the literature for other bacterial species which says that cells without MreB grow in an unregulated fashion, meaning that size and shape are no longer uniform, an observation made here for *B. pertussis*. The difference is that in other bacteria growth was obtained only with a secondary mutation or using minimal media. An *mreB* mutant of *B. pertussis* is viable in standard conditions without a secondary mutant. The mutation only becomes lethal on modulation to the Bvg- phase.

4.1.12 Bvg state of BP536 affects the MIC of ampicillin on charcoal agar

The products of the operon *mreBCDmrdAB* are involved in maintenance of the peptidoglycan cell wall; indeed MrdA is a penicillin-binding protein 2, which is particularly important in synthesising the cell wall along the cell axes. The MIC for ampicillin, an inhibitor of PBPs, was investigated to see if Bvg- phase *B. pertussis* has

different sensitivities to the disruption of the process of cell wall synthesis. The MIC of *B. pertussis* in Bvg+ phase was around 2.5 times greater than in the Bvg- phase (0.125μg/ml and 0.047μg/ml respectively).

4.1.13 *Bordetella pertussis* has different membrane potential dependent on Bvg phase- use of a *petABC* mutant

The TraDIS data show differences in essentiality of different parts of the electron transport chain with respect to Bvg phase. For example, the genes encoding succinate dehydrogenase (*sdhABCD*) are essential in the Bvg- phase but not in the Bvg+. *petABC*, the operon coding for ubiquinol-cytochrome c reductase is nonessential in both Bvg phases but there were significantly less insertions found in the Bvg- phase than in the Bvg+. This operon is designated "fitness affected" because the mutation of these genes creates a greater fitness cost in one Bvg phase than another, in this case more affected in the Bvg- phase than the Bvg+.

A mutant lacking the *petABC* operon was constructed in order to validate the TraDIS data. Briefly, PCR was used to obtain *petABC* right flanking region and *petABC* left flanking region using the primers listed in Chapter 2. The process was then similar to that used to generate the *mreB* mutant, but a kanamycin cassette was not used, meaning that when selecting for the second recombination event there was no control over whether clones would revert to the wild-type genotype or recombine to make knockout mutants. A number of clones were picked and analysed by PCR to determine which clones were knockout mutants.

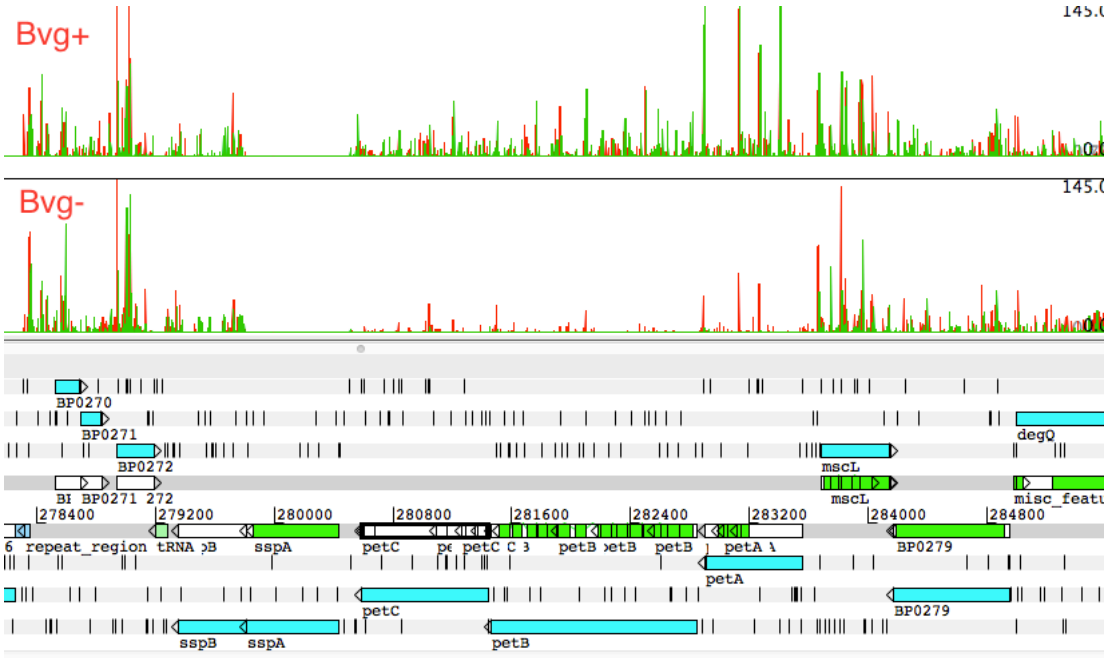


Figure 29– Screenshot of an Artemis plot of insertion sites in and around the *pet* locus for Bvg+ (top graph) and Bvg- (bottom graph) BP536. A snapshot of the genome of Tohama I (from which BP536 is derived) is shown with red and green spikes representing transposon insertion points. The heights of the spikes are proportional to the number of unique insertions recovered at that point in the genome. From this it can be seen that there are fewer insertions recovered from mutants of the *petABC* locus grown in the Bvg- phase than in the Bvg+ phase.

Figure 29 shows the differences between the number of insertions (and thus mutants) recovered for Bvg+ and Bvg- phase BP536. It can clearly be seen that there were fewer mutants recovered in the Bvg- phase meaning that mutants in this phase are significantly less fit than mutants in the Bvg+ phase, and the *petABC* genes all appeared in the list of genes statistically more fitness affected in the Bvg- phase.

4.1.14 There is no growth defect of *pet* mutants compared to BP536

A deletion of the operon *petABC* was made as well as a deletion of only the *petA* gene coding for the Fe-S protein of the ubiquinol-cytochrome c reductase. Both mutants grew well on charcoal agar in Bvg+ and Bvg- conditions, no differently to BP536.

Charcoal agar was the condition under which the TraDIS data was obtained, and no obvious growth defect was observed on charcoal agar in either the Bvg+ or Bvg- phases. The *petABC* mutants were assayed for growth in SS broth compared to BP536 in both the Bvg+ and Bvg- phases (shown in figure 30).

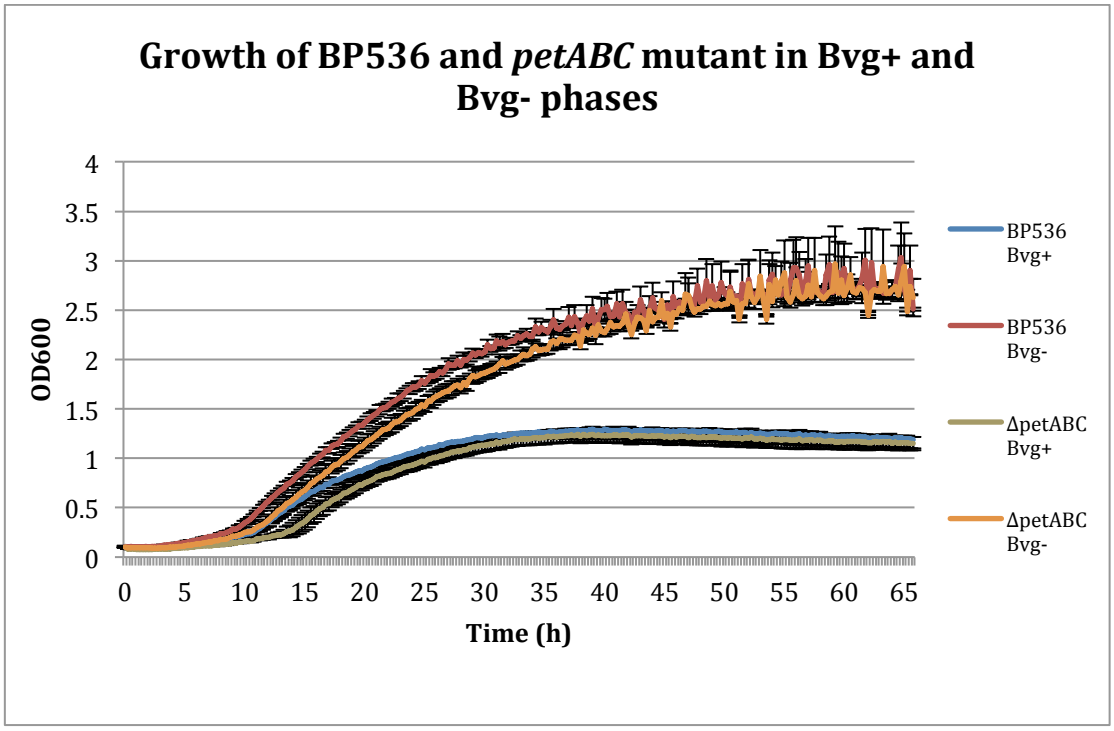


Figure 30- Growth assay of BP536 and *petABC* mutant strains in Bvg+ and Bvg- phases in Bvg+ and Bvg- phases. Each curve is the average of ten replicates. Error bars display standard deviation from the average. Growth was obtained in a 96-well plate and is measured by observing increase in optical density (OD) over time until stationary phase is clearly reached. There is no observable difference in growth between the two strains in either Bvg phase.

There was no difference observed between the two strains grown in the Bvg- phase except for a slight delay in exit from lag phase for the *petABC* mutant in both phases. What is clear is that the *pet* mutants are viable in standard *B. pertussis* liquid broth and grow at least as well as BP536. The fitness costs suggested by the TraDIS data that come with loss of *petABC* genes are not apparent when grown on either charcoal agar or in SS broth.

4.1.15 Proton motive force (PMF) across the membrane in *B. pertussis* is different depending on Bvg phase

Data from the TraDIS experiment suggests that there may be a difference in the electron transport chain dependent on Bvg phase due to the differences in essentiality that are apparent. It is possible that different components of the electron transport chain are used differently in different Bvg phases, and transfer of electrons or pumping of protons could be different between each phase. The PMF of cultures of

BP536 grown in the Bvg+ and Bvg- phases was measured, on two different days, in duplicate both times. Data is presented in figure 31.

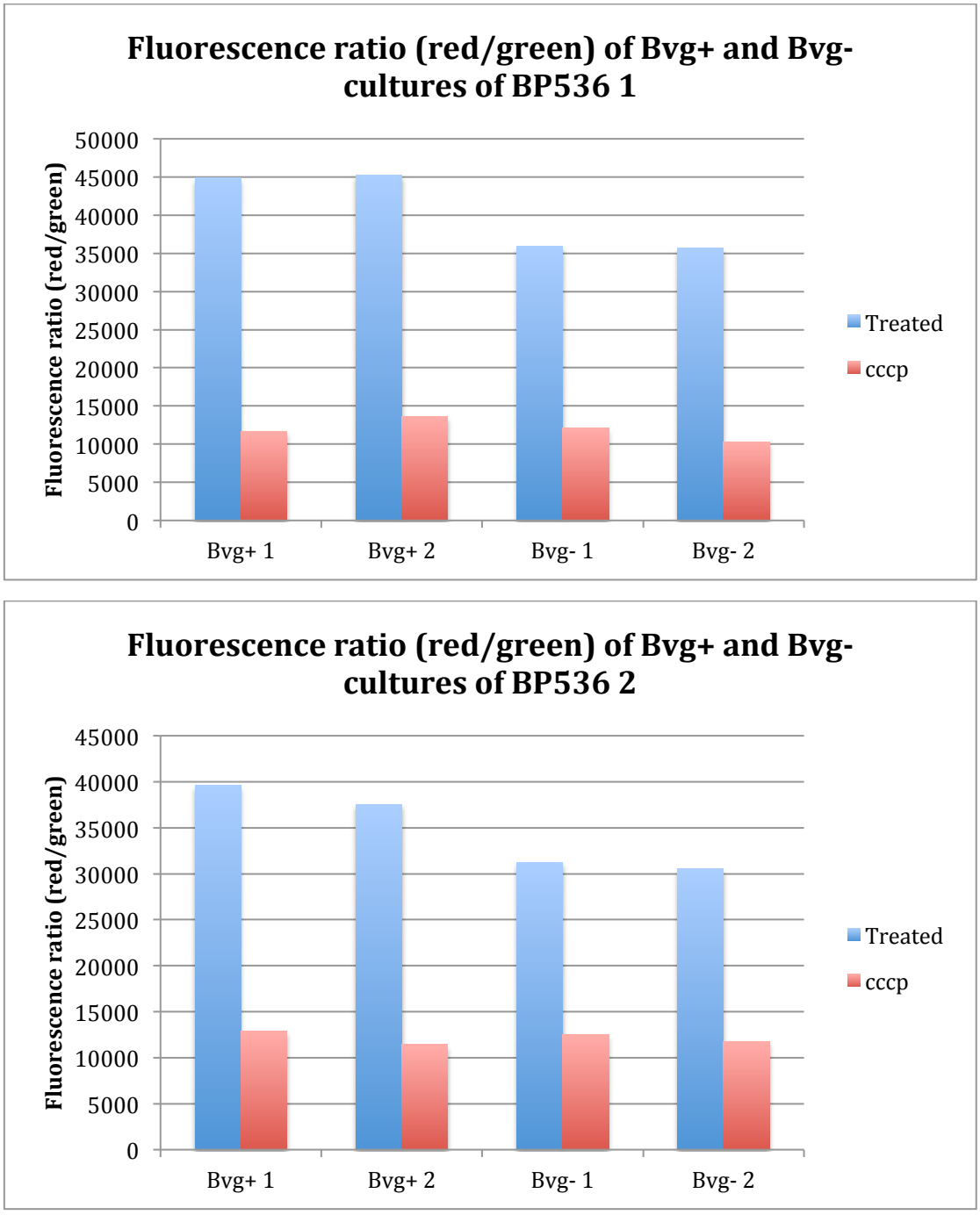


Figure 31- Graphs showing the ratio of fluorescence (red/green) of Bvg+ and Bvg- cultures of BP536. The two graphs are representative of the two experiments done in the same way on different days. Samples treated with $DiOC_2(3)$ are shown as blue bars, while dissipated ($DiOC_2(3)$ + CCCP) samples for each culture are shown as red bars. The height of the bars is fluorescence ratio (red/green) in arbitrary units. Two biological replicates are shown for each Bvg+ and Bvg- condition for each separate experiment.

$DiOC_2(3)$ exhibits green fluorescence, but at higher concentrations in the cytosol of cells the molecules self-associate, and fluorescence shifts to the red end of the spectrum. During the assay the assay molecules of $DiOC_2(3)$ enter the cell in a manner dependent on the PMF. Thus, the ratio of red/green fluorescence is proportional to the PMF. Samples treated in the same way but with carbonyl cyandide 3-chlorophenylhydrazone (CCCP) added are used as a control as CCCP dissipates the PMF, allowing elucidation of fluorescence shift that is dependent on PMF.

Figure 31 shows that the Bvg+ samples have a larger PMF than the Bvg- samples. It is clear that while this result is consistent between both experiments performed on different days, the values for fluorescence ratio themselves are not. To account for day to day variation the ratio Bvg+:Bvg- PMF was calculated for each experiment. This was done by subtracting the values for the CCCP controls from those of the samples and dividing the Bvg+ fluorometric values by the Bvg- values. The four different samples (two Bvg+ and two Bvg-) on one day were from four independent cultures the values for the two Bvg+ samples were both divided by each of the Bvg- samples to give four values for fold-difference between Bvg+ and Bvg-. An average of the four values was calculated. This was done for each experiment performed on the two days (figure 32).

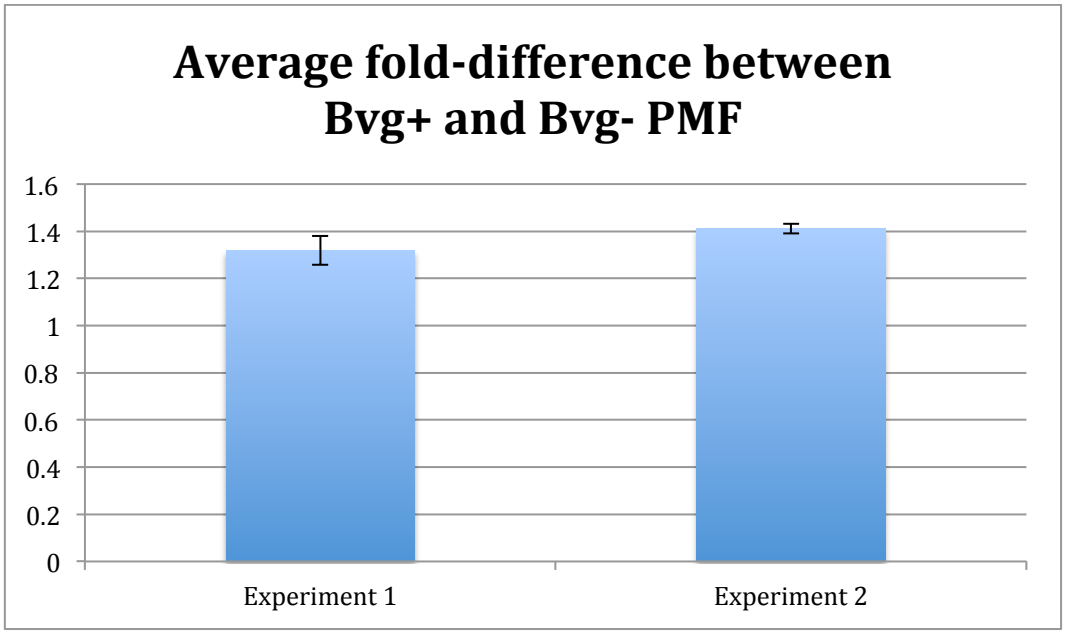


Figure 32- Average ratio of Bvg+ phase PMF to Bvg- phase PMF. The difference between the fluorescence of Bvg+ and Bvg- cultures was calculated for each experiment performed on two separate days. This was to look for consistency between experiments as experiment 1 and 2 were performed on different days. Error shown is standard deviation.

For experiment 1 the Bvg+ samples had an average 1.32 times greater PMF than the Bvg- samples, for experiment 2 they were 1.41 times greater. The difference between these two experiments was not significant as shown by t test ($p=0.052$) when all of the values for ratio Bvg+:Bvg- across the two days are compared, showing that the fold-difference between the PMF of Bvg+ and Bvg- samples is consistent between repeats of the experiment.

Taken together these data show that there is a difference between the PMF measured across the membrane of BP536 dependent on Bvg phase and that the fold-difference in PMF is between 1.32 and 1.41 times greater in the Bvg+ phase than the Bvg- phase.

4.1.16 PMF across the membrane is different dependent on Bvg in a *pet* mutant but the PMF of the mutant is not significantly different from BP536

The operon *petABC* codes for proteins that make up the ubiquinol-cytochrome c reductase complex of the respiratory electron transport chain. This complex couples transfer of electrons to a cytochrome c oxidase with movement of protons across the membrane, contributing to the PMF. It might be expected then that a *petABC* mutant

would generate a lower PMF than BP536. The PMFs of the *petABC* mutant and BP536 grown on charcoal agar in Bvg+ and Bvg- conditions were measured (figure 33). Charcoal agar was used in this case to mimic the conditions under which the TraDIS experiment was performed.

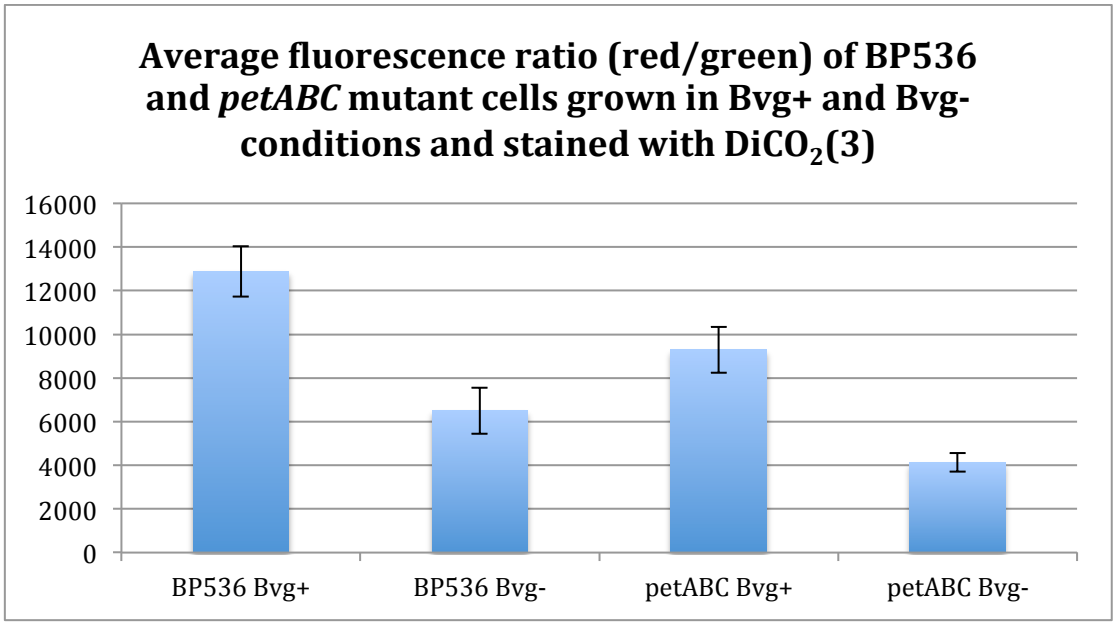


Figure 33- Average fluorescence ratio (red/green) of wild-type and *petABC* mutant grown on charcoal agar. Fluorescence ratio (red/green) is measured in arbitrary units, with the height of each bar being proportional to the PMF produced in cells measured. Averages from 3 biological replicates are shown and values were calculated by subtracting the dissipated (CCCP) values from the treated samples and calculating the mean of triplicate samples. Error bars are standard error of the mean.

There was a significant difference in PMF between BP536 plate cultures grown in Bvg+ and Bvg- phases (12880.33 and 6505, p=0.015) and also for the mutant grown in Bvg+ and Bvg- phases (9289 and 4125.67, p=0.026). Also, there was a difference between PMF in the BP536 versus the mutant, though this wasn't significant in the Bvg+ phase (12880.33 and 9289, p=0.083) or the Bvg- phase (6505 and 4125.67, p=0.141).

The higher PMF of Bvg+ phase BP536 compared to Bvg- phase observed in SS broth was replicated by growth on charcoal agar, and this difference was also observed for the mutant. As noted above, the *petABC* operon codes for a protein complex involved in generating the membrane potential and it is interesting to see that in both phases the *petABC* mutant generates less membrane potential than the wild-type, though in neither of these phases is this difference significant by t-test.

The original observation from the TraDIS was that mutations in the *petABC* genes affected fitness more in the Bvg- phase than the Bvg+. It is possible that the Bvg+:Bvg- ratio of PMF in BP536 is different to that of the mutant, i.e. the PMF of the mutant is more affected in one phase than the other. The Bvg+:Bvg- ratios for the PMF in BP536 and the *petABC* mutant were calculated and are shown in figure 34.

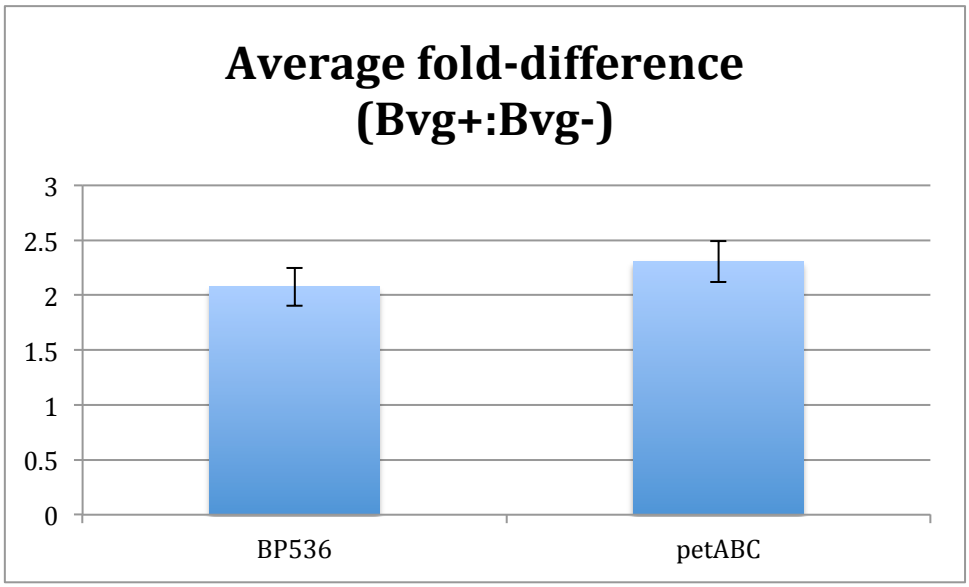


Figure 34- Average fold-differences between the Bvg+ and Bvg- cultures of BP536 and *petABC* mutant. The fold-difference between fluorescence values obtained for cells grown in the Bvg+ and Bvg- phases are shown to discern if there is a similar difference between the PMF of cells grown in the Bvg+ and Bvg- phases between BP536 and the *petABC* mutant. Error shown is standard deviation.

For BP536 and *petABC* mutant samples there were three biological replicates. The fluorometric ratio values of the CCCP controls were subtracted from the fluorometric ratios of samples. The fold-difference between Bvg+ and Bvg- samples were then calculated by dividing the fluorometric value for each Bvg+ sample by each of the three Bvg- samples, giving nine values for fold-difference. These nine values were averaged giving an average fold-difference of PMF between Bvg+ and Bvg- cultures. This was done for BP536 and the *petABC* mutant.

The PMF of plate cultures of Bvg+ phase BP536 is on average 2.08 times higher than that of Bvg- phase BP536. This is more than the 1.32-1.41-fold difference of BP536 grown in SS broth. The PMF of the *petABC* mutant grown on charcoal agar in Bvg+ conditions is 2.31 times higher than when grown on charcoal agar under

Bvg- conditions. This was not significantly higher than the 2.08-fold difference seen for BP536 (p=0.379).

Taken together, these results suggest that BP536 has a 1.32-1.41-fold higher PMF when grown in the Bvg+ phase than in the Bvg- phase in SS broth. This larger PMF of the Bvg+ phase was also observed when the bacteria were grown on charcoal agar, but was measured as 2.08 time greater. The PMF of the *petABC* mutant was lower than the PMF of the wild-type strain in both the Bvg+ and Bvg- phases, but the difference was not significant in either condition. The PMF of the mutant was on average 2.31-fold higher in the Bvg+ condition, more than the 2.08-fold difference seen for the BP536. However, this difference was not significant either suggesting that the mutant produces a marginally decreased PMF with respect to wild-type, this is decreased by the same amount in both Bvg phases and the PMF is not more affected by loss of *petABC* in one Bvg phase over another.

4.2 Discussion

4.2.1 TraDIS

TraDIS was carried out on both the Bvg+ and Bvg- phases of *B. pertussis*, with the aim of using gene essentiality predictions to discern novel physiological features of the two phases. Different growth phenotypes have been described (see Chapter 3) for Bvg+ and Bvg- phase BP536, which show that cells in different Bvg phases are metabolically different. Sequencing-based techniques such as TraDIS mean that data about the entire genome is gained, and specific genes or pathways can be attributed to phenotypic differences seen. Thus, the TraDIS data was used to provide a genetic basis to observations described in Chapter 3.

TraDIS reveals genes that are essential for growth in a particular condition, which includes genes for essential metabolic processes, DNA replication, transcription, genes for ribosomal maturation and peptidoglycan synthesis. It would be interesting to perform TraDIS on cells grown in defined media, so that it is known exactly what the cells have to use for growth and therefore everything that they need to synthesise. In that case one might expect most genes involved in pathways for amino acid biosynthesis, for example, to be essential for growth. However, the experiment requires that cells be plated to allow colonies to grow, and the commonly

used agar media for growth of *B. pertussis* are complex media, containing peptone, starch and other complex macromolecules. In this case it is probable that cells can use substrates in the media to synthesise macromolecules, rather than synthesising all building blocks from a single carbon source as in the case of the defined SS medium. SS agar would be ideal for performing TraDIS, if not for the failure to isolate single colonies on this medium. However, TraDIS still reveals a lot about the biology of the organism. There are metabolic genes that are essential for growth on complex media, including genes coding for parts of the electron transport chain, the TCA cycle and gluconeogenesis.

Genes for which mutants are less fit in one Bvg phase than another are labelled fitness affected. If there are significantly less read counts for a gene in one phase than the other (p value<0.05) this gene is called fitness affected in that phase. This label adds an extra layer on top of genes simply being conditionally essential or not, since if a mutant of a gene is less fit in one phase over the other this also provides biological information about the Bvg phase in question.

TraDIS is a quantitative technique in which read count is used to determine the "essentiality" of a gene. Cut-offs must be set in order to label a gene as essential, or non-essential. Some genes will be on the edge of cut-offs and are designated ambiguous. Ambiguous means neither essential nor non-essential, but biologically genes have to be one or the other. This is a caveat of TraDIS. Genes that are essential in one phase might be ambiguous in the other meaning they may not be truly differentially essential but essential in both phases. Conversely this may not be the case and the gene may actually be differentially essential. This is one reason why specific genes of interest may need to be further validated by making the mutant and testing the growth phenotype. Clearly, however, for an experiment dealing with such a large number of genes, cut-offs need to be drawn somewhere and at least providing some idea of ambiguity gives an idea of the scale of essentiality from completely essential to strong fitness cost to non-essential.

4.2.2 TraDIS reveals conditionally essential genes, dependent on Bvg phase

The main objective of the experiment was to reveal gene that are essential for growth in both the Bvg+ and Bvg- phases of *B. pertussis*. There are 79 genes that are essential only in the Bvg- phase, a lot more than the 19 that are essential only in the

Bvg+ phase. This reveals something about the growth constraints with respect to Bvg phase. In particular that *B. pertussis* has different sensitivities to the loss of genes dependent on Bvg phase.

There are a number of gene categories assigned to the conditionally essential genes, but particular attention has been paid to genes involved in metabolic processes or cell growth, including cell wall biosynthesis. Of the genes that are essential for growth only in the Bvg- phase there are more involved in pathways of cell wall synthesis and energy metabolism (i.e. the electron transport chain) than in the Bvg+. This reveals the different essentiality of these processes depending on Bvg phase. Since cells in the Bvg- phase divide more often than the Bvg+ cells it is probable that they have to grow more quickly to reach the point at which cell division is triggered. This would mean that mutants in genes involved in peptidoglycan synthesis, a crucial part of cell growth, would be less fit or non-viable.

There are a number of genes that are involved in the electron transport chain that are differentially essential, including all four genes coding for the succinate dehydrogenase complex, which is essential only in the Bvg- phase. Furthermore, the *petABC* operon coding for the cytochrome c reductase complex, is more fitness affected in the Bvg- phase. This underlines the importance of a fully functional electron transport chain in the Bvg- phase. In the Bvg+ phase the only essential components are the NADH dehydrogenase and the ATP synthase, while all components are essential or fitness affected in the Bvg- phase except the terminal oxidases.

4.2.3 *mreB*: validation of the TraDIS

As discussed above, it is desirable to validate observations of particular interest made by TraDIS. To this end a mutant of *mreB* was made and was found to be conditionally essential for growth on charcoal agar dependent on Bvg. This validates the TraDIS result. To my knowledge this is the first example of a mutant of *mreB* being conditionally viable dependent on activity of a two-component system.

MreB is a bacterial homologue of actin. It forms filaments of up to 3.4μm long with an average width of 75nm. These filaments range in size and are highly dynamic, moving around the cell seemingly rapidly assembling and disassembling [175]. MreB is involved in maintaining organisation inside the cell, and has been implicated in protein transport within the cell [176]. Also, it is linked to the cell wall synthesis

machinery, playing a role in the positioning of membrane proteins MreC, MreD and RodZ, the loss of any of which has been reported to lead to loss of shape in rod cells which become spheroid and die [166, 175]. Thus expression of the *mreB* gene is considered to be essential for growth. The reasons why cells with inadequate levels of MreB die have been discussed previously and it has been reported that *E. coli* cells lacking MreB were conditionally viable, that is they grew as small dividing spheres on minimal media but growth on complex media produced giant nondividing spheroids [166, 177]. The lethality of these *mreB* mutants could be partially overcome by the supply of an overactive form of (p)ppGpp synthase or by increased expression of FtsZ, the protein that forms the septal ring. Also, it was also noticed that in the mutants there was an extension and invasion of the cellular membrane, possibly arising from an excess membrane in the interior of *mreB* mutants, because the mutants synthesised phospholipids at the same rate as the wild-type, but failed to adjust for new surface requirements of maintaining growth of larger cells. There was an aberrant assembly of FtsZ and MinE on these internal membranes and thus an interference with the proper assembly of the septal ring for cell division.

Cell death due to loss of expression of *mreB* is caused by the membrane being synthesised at an unchanged rate in spheroid cells, faster than is required. This excess membrane forms vesicles in the cytoplasm, which compete for FtsZ, preventing it from initiating normal division. This is why overexpressing *ftsZ* can reverse the lethality of loss of MreB [177]. In addition to supplying extra FtsZ, it was found that *mreB* mutants were able to survive and propagate in conditions of slow mass increase, either at lower temperatures or on minimal media [166]. Unlike in these systems, *B. pertussis mreB* mutant was differentially essential based on the activity of a two-component system, Bvg growing on the same complex medium.

The observation that the *mreB* mutant of *B. pertussis* has no growth defect in SS broth is surprising. SS broth, however, is a different medium to charcoal agar. It may be the nutrient differences between the media changed the requirements for *mreB,* although cells still grew as spheres so the gene is required to produce rod-shaped cells. It may be that growth on solid media is what is important to observe a lethal effect of *mreB* mutation in *B. pertussis. B. pertussis* growth is slower than *E. coli* so it may be that growth in SS broth is slow enough to allow growth of cells in this condition regardless of Bvg activity, although growth in liquid media is not

expected to be slower than growth on solid media, which typically takes 3-4 days for visible single colonies to form.

The observation that heptakis was required for growth of the *mreB* mutant in SS broth is interesting. Heptakis is known to improve the growth of *B. pertussis* by sequestering the free fatty acids produced during growth [120] This suggests that the *mreB* mutant is more sensitive to the effects of these free fatty acids than BP536, possibly because since there is a defect in the cell wall the mutant cells are more easily compromised by lipids.

TEM images show that mutants of *mreB* are larger in width, length, and volume. The shape of these cells is spherical. By contrast, BP536 cells are smaller and more uniformly rod-shaped. A spherical cell shape for *mre* mutants has been described before in other bacteria, and the basis for *mreB* originally being described as a rod-shape determining gene. *mrdA*, being involved in elongation of the cell wall, plays a key role in forming the elongated rod shape of the cell. Since *mreB* has been described as a dynamic scaffold that provides spatial regulation to cell synthesis machinery by holding in place, this gene too has a key role in regulating the cell shape. Cell size is normally tightly regulated and uniform, and is linked to the cell division, cells usually growing by a set amount before dividing [150, 151]. Thus *mreB* is required for proper regulation of cell size and shape, and without it cells grow in a non-uniform fashion, and are larger than BP536 cells.

mreB was one gene chosen out of an operon of five genes (*mreBCmrdAB*) the products of which are thought to work together to coordinate cell wall synthesis along the axis. *mrdA* codes for penicillin-binding protein 2, which has transpeptidase activity and is involved specifically in synthesising the cell wall along the axis of the cell [170, 178, 179]. Mutants of this gene also lose their rod shape revealing a role for this gene in regulating the shape of rod cells, specifically by synthesising peptidoglycan to elongate the cell [180]. Since mutants of this operon are nonviable in the Bvg- phase this could be said to be an essential process for the Bvg- phase.

Another way of disrupting normal cell wall synthesis is by inhibiting the process, for example by using β-lactam antibiotics to inhibit PBPs. When the MIC of *B. pertussis* to the β-lactam ampicillin was investigated it was found that although growth in both Bvg phases is sensitive to ampicillin, growth in the Bvg+ phase is less sensitive. This provides further evidence to the claim that the Bvg- phase is more sensitive to changes in normal, coordinated cell wall synthesis. It has been reported

previously that the Bvg locus may have an effect on rate of bacterial killing by β-lactams and cell wall degradation [181] and it was suggested that this could be due to inhibition of autolysins making *B. pertussis* in the Bvg+ phase more phenotypically tolerant of antibiotics, though it was also noted that no difference was observed between the Bvg+ and Bvg- *B. pertussis* in MIC to β-lactams. It is not clear if this is due to different requirements for PBPs, or more of one PBP being present in cells of one phase over the other. Another explanation for this observation could be that growth rates differ between the two phases and generally faster growing bacteria are more susceptible to antimicrobials than slower growing, but it would be difficult to show that this is true on solid medium.

MreB is in a complex with genes involved in cell wall synthesis, such as *mrdA*, PBP2, which are involved in synthesising peptidoglycan in an elongated fashion. Since loss of *mreB* leads to the lack of ability to regulate cell shape and size, and since cell shape and size are linked to regulation of cell division, it is likely that *mreB* mutants are impaired in their ability to regulate cell division. The observation that *mreB* and the wider operon are essential for growth in the Bvg- phase fits with the picture from growth data indicating that faster growth along the cell axis is a fundamental part of growth in this phase and provides evidence that this is how cells in this phase grow faster and divide more often.

4.2.4 Use of TraDIS to discern information about the electron transport chain of *B. pertussis*

There were differences between the essentiality of different parts of the electron transport chain between the Bvg+ and Bvg- phase. Genes for the NADH-dehydrogenase complex and the ATP synthase are essential in both phases, while genes for the cytochrome c oxidase are non-essential. The *sdhA-D* genes, which code for succinate dehydrogenase are essential in the Bvg- phase, and mutants of *petABC*, coding for the cytochrome c oxidoreductase are more fitness affected in the Bvg- phase. This means that overall the Bvg- phase is more sensitive to disruption of the electron transport chain.

To investigate this further, a deletion of the *petABC* operon was made. These genes code for complex III, which functions as part of the electron transport chain, oxidising ubiquinone, reducing cytochrome c_1 and releasing protons that contribute to

the proton motive force. Contrary to the result from the TraDIS experiment, which suggested that mutants of this locus would be fitness affected depending on Bvg phase, there was no difference in growth between the mutant and BP536 in SS broth. It should be noted, however that the TraDIS was carried out with selection on charcoal agar and not broth, although it is difficult to measure growth on solid media. It is possible that other complexes that contribute to the proton motive force compensate for the loss of *petABC* when these mutants are grown in isolation and exhibit no growth defect, but when grown in competition with a strain that expresses a normal electron transport chain the mutant is outcompeted.

Without *petABC* it could be expected that fewer protons would be pumped out of the cell and a smaller PMF would be generated. Firstly, however, the PMF of BP536 was investigated in the Bvg+ and Bvg- phases and it was found that the PMF of the Bvg+ phase was between 1.32 and 1.42 times higher than in the Bvg- phase. This is perhaps surprising since the Bvg+ phase grows slower and to a lower maximal yield. However, if in the Bvg+ phase cells are consuming more glutamate to fuel a more active TCA cycle and excess carbon is released as carbon dioxide then a result of this would be greater production of NADH for the electron chain and possibly higher activity of the electron transport chain as a whole, resulting in a higher PMF. This would explain why mutants of *petABC* have a higher fitness cost in the Bvg- phase, since mutating a complex that contributes to the PMF would have a greater impact in cells that generate a smaller PMF.

The difference between the PMF of Bvg+ and Bvg- phase cells was more pronounced when the cells were cultured on plates, although the PMFs generally appeared to be lower. This may be a result of the microaerobic environment of agar plates resulting in lower activity of the electron transport chain due to a lesser availability of oxygen as a terminal electron acceptor than when cultures are grown in flasks.

The PMF generated by the *petABC* mutant was lower than BP536 in both phases, which is to be expected since the complex contributes protons to the PMF. However, this difference was not significant by T test, which shows that while *petABC* does contribute to the PMF, this complex would not be considered fundamental for maintenance of the PMF. The comparison of the PMFs between BP536 and the *petABC* was carried out on cells that had been cultured on plates, which already causes a lower PMF to be generated than cells grown in flasks. It may

be that growing cells in flasks and comparing the PMFs of BP536 and the mutant would result in a more significant difference. It could also be that in the absence of the proton pumping cytochrome bc_1 complex that cells simply respond by increasing the activity of the other proton pumps so while the PMF is decreased in the *petABC* mutant, cells are still viable and without a growth defect.

The difference between the PMF of cells grown in the Bvg+ and Bvg- phases is also apparent for the *petABC* mutant. The fold-difference between the PMFs of the two Bvg phases for the mutant is similar to that for BP536, showing that the reason why the PMF is higher in the Bvg+ phase of *B. pertussis* is not due to activity of the cytochrome bc_1 complex, and is probably because of general increased activity of the electron transport chain.

The TraDIS data was used to inform about differences in the biology of *B. pertussis* grown in the Bvg+ and Bvg- phases, and led to the observation that the parts of the electron transport chain were differentially essential for growth. Furthermore, because of information gain by TraDIS data, the PMF of Bvg+ phase BP536 was shown to be significantly higher than the Bvg- phase. This is interesting, especially when seen in the light of data showing that in the Bvg+ phase BP536 consumes glutamate at a faster rate than in the Bvg- phase, and possibly has a more active TCA cycle. This would make sense given that a more active TCA cycle would provide more NADH, which would be used as a substrate to generate more of a PMF in the Bvg+. The reasons why Bvg+ phase *B. pertussis* would consume more glutamate to have a more active TCA cycle and have a more active electron transport chain to generate more of a PMF are not clear, especially given the observations that in this phase fewer cells are made and less biomass produced. However, one could assume that if a higher PMF is being produced, it is possible that more ATP is being synthesised in the Bvg+ phase, though what this would be used for is not clear.

Chapter 5- RNAseq reveals novel elements of the Bvg regulon

5.0.1 RNAseq

Gene microarrays are a tool used to investigate global gene expression without next-generation sequencing. DNA oligos are ordered on an array with different genes occupying different spots on the array. cDNA is synthesised from RNA samples and fluorescently labelled. cDNA from two different samples can be differentially labelled, for example one can be green and the other red. The two samples are mixed and hybridised to the array. The fluorescence is then measured, the more red or green the fluorescence the more mRNA was expressed from one sample over the other. This requires knowledge of the sequences of the features monitored by the array. Another disadvantage is that non-specific cross-hybridisation and fluorescence occur even in the absence of target DNA can lead to a high background level of fluorescence [182]. Quantification is typically relative between two conditions rather than being absolutely quantitative.

The advent of next generation sequencing has paved the way for more advanced methods of investigating transcriptomes. Typically, RNA is used to synthesise cDNA, which is then sequenced. Adapters are ligated onto the RNA molecules, and they are sequenced using short reads either from one or both ends of each molecule. This is RNAseq [183]. RNAseq required no prior knowledge about the genome of interest and following sequencing the reads can either be aligned to a reference or assembled, thus RNAseq is not limited by genomic sequence data being available already. RNAseq is more sensitive than hybridisation methods in that even very small amounts of expression can be detected. There are no issues with background or saturation seen with fluorescence, and RNAseq can be used to measure a large range of expression changes. Sequencing technologies can provide deep coverage of transcripts, meaning even rare transcripts can be detected, and the accuracy of sequencing can provide resolutions of 1bp, meaning that transcripts can be accurately mapped to existing genomes revealing exon or gene boundaries. RNAseq requires no prior knowledge of a genome seuqecne as reads can assembled *de novo* or mapped to a reference. RNAseq can be applied to organisms with complex transcriptomes, for example where splicing occurs, or to prokaryotes where poly-A tails are absent and splicing doesn't occur. Thus RNAseq provides a higher-

throughput, more accurately quantitative method of performing genome-wide expression studies, which can also reveal precisely where transcripts start and end, and which can be applied to any organism regardless of what prior genomic information is known.

5.0.2 Microarray studies in *B. pertussis*

Expression studies can reveal much about the biology of *B. pertussis*. Microarrays were used to show that genome rearrangements may contribute to gene expression profiles in strains that are otherwise identical at the genome level [184]. Another microarray study compared gene expression profiles of *B. pertussis* in normal growth conditions to conditions of iron starvation and identified a novel system important in the use of siderophores [185]. A transcriptomics approach, the term used for genome-wide expression studies, was also taken to find that *ptxP3* strains of *B. pertussis* expressed virulence genes other than pertussis toxin more highly than *ptxP1* strains. This was also done by microarray [186]. Furthermore, microarray was used to show that RNA chaperones play a role in expression of virulence factors in *B. pertussis* [187].

Microarray was used to define the Bvg regulon of *B. bronchiseptica* and *B. pertussis* [143]. Virulence factors comprised a major part of the genes maximally expressed in the Bvg+ phase, while a number of genes maximally expressed in the Bvg- phase encoded proteins involved in protein folding, ushering and transport. Further genes more highy expressed in the Bvg+ included autotransporters, genes for iron acquisition, as well as potential new virulence genes. Capsule biosynthesis genes were the most strongly Bvg-regulated that were more highly expressed in the Bvg- phase, while in *B. bronchiseptica* genes for chemotaxis and flagellar biosynthesis were also more highly expressed in the Bvg- phase.

The Bvg- phases of *B. pertussis* and *B. bronchiseptica* displayed only weak similarity, as only 13 genes more highly expressed in this phase were common to both species. Genes involved in the TCA cycle were more highly expressed in the Bvg- phase in *B. bronchiseptica*, as were transporters for amino acids and C_4-dicarboxylate transporters. It was speculated that this might lead to increased levels of TCA cycle intermediates as well as amino acids that could be metabolised to acetyl-CoA and used to feed into the TCA cycle. Expression of other genes suggested greater activity

of the urea cycle, which also feeds into the TCA cycle. All in all it was thought that in the Bvg+ phase there might be a difference in preference for carbon and nitrogen source, for example lactate, glutamine and ammonia over other amino acids. In this way physiological differences were inferred from identifying particular gene classes that were differentially expressed in different conditions. In another example, genes encoding NADH dehydrogenase, lactate dehydrogenase and a cyctochrome c were upregulated in the Bvg+ phase; while genes for two cytochromes, two ferrodoxins and a cytochrome bc1 complex were upregulated in the Bvg- phase suggesting different preferences for the activity of the electron transport chain.

Metabolism was not so obviously Bvg regulated in *B. pertussis* as in *B. bronchiseptica*. The only Bvg+ phase genes shared between the two species were the ammonia transporter and genes for haem biosynthesis. The *B. pertussis* homologues of transporter and cytochrome genes that were Bvg-regulated in *B. bronchiseptica* were not Bvg regulated in *B. pertussis*.

There were many differences in Bvg regulated genes between strains of *B. pertussis* and only 23 out of the 113 Tohama I Bvg- genes were regulated in another strain. *vrg6, vrg18, vrg 24* and *vrg73* were more highly expressed in the Bvg- phase only in Tohama I. 60% of the genes upregulated in the Bvg+ phase were similarly regulated between the two strains. This suggests a difference between the importance of the Bvg+ and Bvg- phase in *B. pertussis* since genes maximally expressed in the Bvg- phase are less conserved between strains than those more highly expressed in the Bvg+ phase. These genes with conserved regulation were mostly involved in virulence, protein folding and transport and most of the known virulence genes were similarly upregulated in the Bvg+ phase, again suggesting that conservation of regulating virulence is important for *B. pertussis*, but that expression of some of the Bvg- phase genes less so.

RNAseq can help refine microarray data, giving a more accurate and complete picture of the Bvg regulon. The advantages come from the precision mapping of transcripts to within 1bp that enables definition of where a transcript begins and end, and not having the high background associated with fluorescence. Another advantage is that RNAseq reveals a previously hidden level of regulation by small non-coding RNAs (sncRNAs), as these can be sequenced along with mRNAs. These have already been shown to be important in regulation of genes in *B. pertussis* [187, 188].

5.1 Results

5.1.1 Validation of the RNA used for RNAseq

RNAseq was used to provide further evidence for some of the observations and proposed models discussed so far. The technique provides a quantitative measurement of expression differences for every gene in the genome between the Bvg+ and Bvg- phases in one experiment. Thus RNAseq can be used to back up data already discussed, for example if genes involved in metabolism, cell wall synthesis and genome replication are differentially regulated between Bvg phases of *B. pertussis*.

RNA for RNAseq was validated by real-time quantitative PCR (q-PCR) to check for expected expression of two genes in the Bvg+ phase and lower expression in the Bvg- phase. cDNA was synthesised to RNA and qPCR was performed to measure relative expression levels of the *fhaB* and *ptxA* genes. Analysis was carried out using the $2^{-\Delta\Delta Ct}$ method [189], *adk* and *tyrB* were used as two independent "housekeeping" standards.

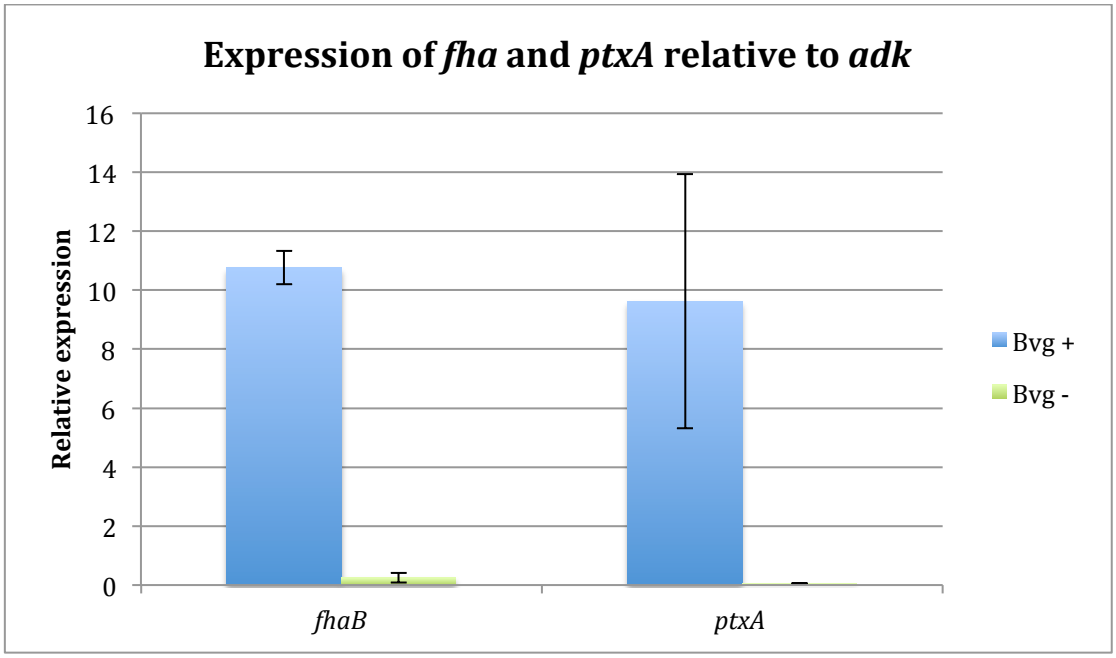


Figure 35– Expression levels of *fhaB* and *ptxA* relative to the expression of the *adk*. Date shown is expression of *fhaB* and *ptxA* relative to *adk*, a housekeeping gene believed to not change expression dependent on Bvg. The data depicted show a level of expression of the *fhaB* and *ptxA* genes in the Bvg+ phase, which is not seen in the Bvg- phase. Data is an average of 3 biological replicates for each Bvg condition. Error is standard deviation.

The RNA was validated by qPCR to check that cells from the cultures grown to purify the RNA had been in the correct Bvg phases, specifically that modulation by addition of 50mM $MgSO_4$ did induce the Bvg- phase and that when grown in non-modulating conditions cells grew in the Bvg+ phase. Expression levels of *fhaB* and *ptxA* were measured because these are known Bvg- regulated genes [133, 190]. The gene for adenylate kinase (*adk*) was used as a standard by which the relative expression of the other two genes was measured because it is not known to be Bvg regulated. Figure 35 shows that the cultures grown for isolation of the RNA highly expressed *fhaB* and *ptxA* in the Bvg+ but that expression was downregulatd in the Bvg- phase.

Another standard gene, *tyrB* was used, also not known to be Bvg regulated, to make sure the same pattern was observed (figure 36).

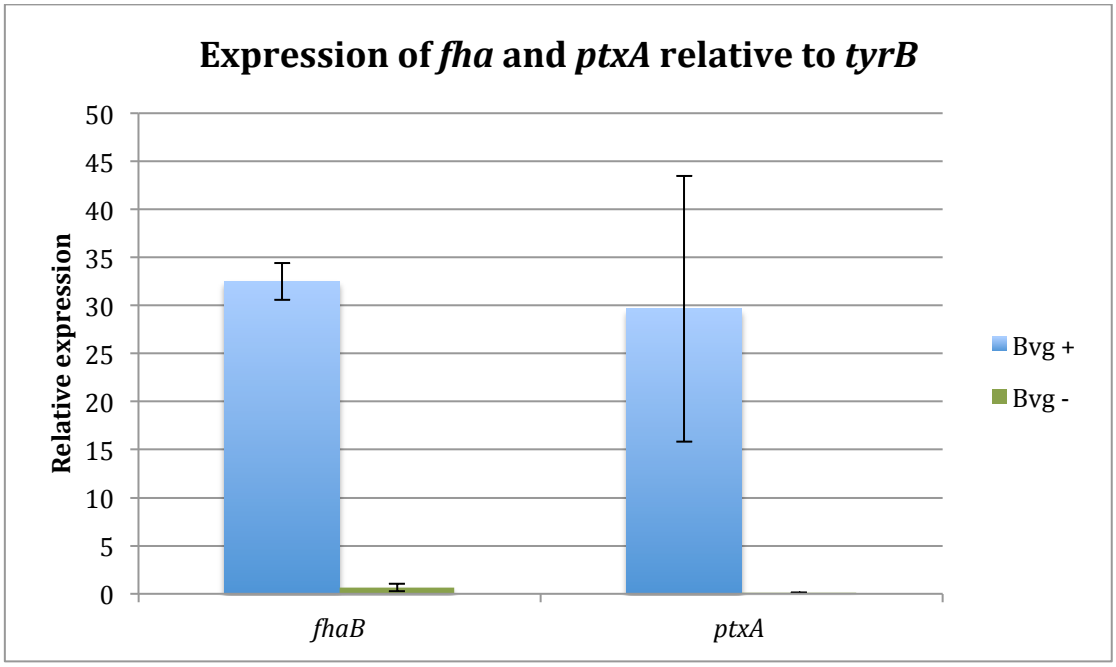


Figure 36– Expression levels of *fhaB* and *ptxA* relative to the expression of *tyrB*. Similar to figure 35 expression of the *fhaB* and *ptxA* genes are shown relative to a housekeeping gene that is not expected to be under the control of Bvg, in this case *tyrB*. Data shown is an average of 3 biological replicates, error is standard deviation.

The observation that the cultures expressed *fhaB* and *ptxA* at a much higher level in the Bvg+ phase than in the Bvg- phase relative to two different standard genes was vital in validating the Bvg conditions used to grow the cultures for RNAseq.

5.1.2 RNAseq- Most highly regulated genes in Bvg+ and Bvg- phases

Genes with a more than 2-fold expression difference between the Bvg+ and Bvg- phases of *B. pertussis* were considered to be Bvg-regulated. There were 531 genes with more than a 2-fold expression difference and of those 248 were more highly expressed in the Bvg+ phase with 283 more highly expressed in the Bvg- phase. Of those 37 and 60 were pseudogenes or copies of the IS element transposase, leaving 211 genes that were more than 2-fold more highly expressed in the Bvg+ phase and 233 genes that were more than 2-fold more highly expressed in the Bvg- phase. The top thirty most highly Bvg-regulated genes in each Bvg phase are shown in table 7.

Bvg+			
Gene	**Product**		**Fold change**
BP2924	putative exported protein	Unknown	183.73
BP2925	conserved hypothetical protein	Unknown	114.23
prn	pertactin precursor	Virulence (surface adhesin)	97.40
BP3792	putative bacterial secretion system protein	Virulence (T4SS)	78.95
fimD	fimbrial adhesin	Virulence (surface adhesin)	76.94
ptxD	pertussis toxin subunit 4 precursor	Virulence (toxin)	71.90
fhaB	filamentous hemagglutinin/adhesin	Virulence (surface adhesin)	70.80
BP2936	putative exported protein (1TM domain) Me t'ase	Methyltransferase	70.38
fimC	outer membrane usher protein precursor	Virulence (surface adhesin)	63.81
brpL	putative RNA polymerase sigma factor	Transcription	59.13
BP3791	putative membrane protein	Virulence (T4SS)	57.77
bscP	hypothetical protein	Unknown	57.60
tcfA	tracheal colonization factor	Virulence (toxin)	55.67

	precursor		
ptxB	pertussis toxin subunit 2 precursor	Virulence (toxin)	54.45
BP0398	ArnT	Glycosyl transferase (cell envelope biogenesis)	50.77
ptxE	pertussis toxin subunit 5 precursor	Virulence (toxin)	46.94
ptxA	pertussis toxin subunit 1 precursor	Virulence (toxin)	44.16
brkA	serum resistance protein	Autotransporter (complement resistance)	43.68
BP0399	ArnT locus glycosyl transferase	Glycosyl transferase (cell envelope biogenesis)	39.79
BP2926	conserved hypothetical protein	Unknown	37.63
bscO	putative type III secretion protein	Virulence (T3SS)	34.79
BP2927	putative integral membrane protein	Unknown	32.25
ptxC	pertussis toxin subunit 3 precursor	Virulence (toxin)	31.91
bfrD	probable TonB-dependent receptor for iron transport	Iron (siderophore) transport	31.59
fim2	serotype 2 fimbrial subunit precursor	Virulence (surface adhesin)	30.19
sphB1	autotransporter subtilisin-like protease	Virulence (surface adhesin)	27.67
bscE	hypothetical protein	Unknown	23.69
bipA	putative outer membrane ligand binding protein	Unknown	22.19
BP1251	putative toxin	Virulence (putative	21.13

		toxin)	
bscQ	putative type III secretion protein	Virulence (T3SS)	20.38
Bvg-			
Gene	**Product**		**Fold change**
kpsM	putative polysialic acid transport protein	Capsule biosynthesis	119.12
vipC	capsular polysaccharide biosynthesis protein	Capsule biosynthesis	98.71
kpsT	polysialic acid transport ATP-binding protein	Capsule biosynthesis	90.30
wbpP	capsular polysaccharide biosynthesis protein	Capsule biosynthesis	81.22
wza	putative capsular polysaccharide export protein	Capsule biosynthesis	73.42
wbpO	capsular polysaccharide biosynthesis protein	Capsule biosynthesis	62.58
wbpT	capsular polysaccharide biosynthesis protein	Capsule biosynthesis	59.90
kpsE	capsule polysaccharide export inner-membrane protein	Capsule biosynthesis	59.49
wcbA	capsular polysaccharide export protein	Capsule biosynthesis	54.98
BP1620	putative glycosyl transferase	Cell envelope biosynthesis	46.36
BP2782	lipoprotein	Unknown	45.67
BP1619	hypothetical protein (glycosyltransferase?)	Unknown	40.22
BP1738	conserved hypothetical protein	Stress response?	30.09
tviD	putative N-terminal region of capsular polysaccharide biosynthesis protein (partial)	Capsule biosynthesis	28.87
BP1737	putative membrane protein	Unknown	23.83

BP3517	putative membrane protein	Unknown	19.64
BP1617	C-terminal region of a putative polysaccharide biosynthesis protein (partial)	Capsule biosynthesis	18.42
BP1736	putative exported protein	Stress response? (osmotic stress)	17.17
BP2780	putative lactate/malate dehydrogenase	Metabolism	11.51
BP3518	Cardiolipin synthase	Lipid biosynthesis	10.91
BP2148	conserved hypothetical protein	Unknown	10.28
fim3	serotype 3 fimbrial subunit precursor	Virulence (surface adhesin)	10.10
BP2569	putative lipoprotein	Unknown	9.76
BP0546	bacterioferritin	Iron transport/homeostasis	7.43
BP2020	hypothetical protein	Unknown	6.99
BP2803	putative integral membrane protein (acyltransferase?)	Cell envelope biosynthesis	6.91
BP1111	hypothetical protein	Unknown	6.86
vir-18	vir-repressed protein	Unknown	6.50
cpn60	60 kDa chaperonin	Protein folding	6.35
vrg-6	Virulence protein	Virulence?	5.84

Table 7- The top 30 most highly Bvg-regulated genes. Genes more highly expressed in the Bvg+ phase are in the top half of the table, while genes more highly expressed in the Bvg- phase are in the bottom half. Gene names, their annotated descriptions and gene categories are depicted as well as fold change of expression relative to the other Bvg phase.

The Bvg+ genes with the greatest fold difference in expression between phases relative to the Bvg- phase included the classical virulence factors i.e. genes for the pertussis toxin and secretory system, fimbriae, FHA, the T3SS and the Ptl T4SS for secretion of PT. The RNAseq data also shows that both *bvgS* and *bvgA* are upregulated in the Bvg+ phase, as is the repressor *bvgR*, showing autoregulation of the Bvg system. The most highly regulated genes in the Bvg- phase were genes

involved in synthesis and export of the capsule. *fim3* is highly expressed in the Bvg- phase.

These data back up what is already known about Bvg, that it is a regulator of virulence in *B. pertussis*. That genes involved in virulence and capsule biosynthesis were among the most highly regulated genes shows that the data had features that were expected, further validating the conditions used to grow the cultures. However, the data show that the Bvg regulon is more than just a regulator of virulence as many genes coding for proteins involved in stress response, cell envelope biogenesis and metabolism are under control of Bvg, highlighting the role of this system as a global regulator with many functions.

5.1.3 A wider Bvg regulon

There are a number of genes encoding chaperones in the Bvg regulon, at least 6 maximally expressed in the Bvg+ phase, most unannotated, and 7 in the Bvg- phase includin *clpB, dnaK, dnaJ, cpn60, cpn10* and the RNA chaperone *hfq*.

Genes involved in iron uptake were Bvg-regulated. There were two siderophores upregulated in the Bvg+ phase; including *BP1141* and *bfrD*. It has previously been established that during infection *B. pertussis* is iron starved so at least some iron acquisition genes were expected to be Bvg-regulated [191]. Conversely, In the Bvg- phase there were three genes for iron uptake upregulated; *tonB*, *BP0546* and *BP0134* suggesting different ways of scavenging iron are used dependent on Bvg phase. Expression of *tonB* was previously described as Bvg-independent, through use of a *bvgAS* knockout mutant [192].

Transport was the category with the highest number of genes maximally expressed in the Bvg+ phase with at least 43 (19%) of genes upregulated in this phase having some role to play in transport. It was the second largest category for genes upregulated in the Bvg- phase with 35 (16%) falling into this category. The majority of transporters upregulated in the Bvg+ phase coded for probable amino acids transporters and included *livFGH*, *livJ* and *livM,* annotated as branched-chain amino acid transporters. There were four genes thought to be involved in the transport of carboxylates including *smoM*, and one operon of three genes involved in the transport of glutathione, *BP2394-96*, upregulated in the Bvg+ phase. There was at least one gene each involved in the transport of metals, sulphate (*sbp*), phosphate (*pstB*), D-

alanine (*dltB*) and glutamine (*glnQ*) upregulated in the Bvg+ phase. The most striking differences were that there were five genes involved specifically in export in the Bvg- phase that were upregulated in contrast there was only one in the Bvg+ phase. There were also three genes believed to be involved in the transport of metals upregulated in the Bvg- phase, as well as two genes involved in the transport of ectoine, a molecule synthesised to combat osmotic stress.

There were some Bvg-regulated genes that belonged to categories represented only in one Bvg phase. There were three genes coding for autotransporters, including *brkA* and *vag8*, upregulated in the Bvg+ phase, but none in the Bvg- phase. There was one gene more highly expressed in the Bvg+ phase that was involved in the cell division process, but four more highly expressed in the Bvg- phase, including *minE*.

Among the genes more highly expressed in the Bvg- phase are at least 11 genes that are involved in the response to stress. These include ectoine synthesis gene *ectA*, *rpoh*, *clpB, dnaJ* and *osmB*. Two genes involved in flagellar synthesis, *fliH* and *BP0877* are more highly expressed in this phase, although *B. pertussis* does not expressed functional flagella due to multiple gene disruption by insertion elements [1]. This appears to be a remnant from an ancestor of *B. pertussis*, and there are more pseudogenes that are Bvg-regulated that are more highly expressed in the Bvg- phase than the Bvg+, suggesting that there is continued reductive evolution in the Bvg- phase over the Bvg+ and that the Bvg- phase plays less of a role in the lifecycle of *B. pertussis*.

Differentially expressed genes would be expected to inform about the environment under which they would be expressed. For example, genes in involved in the transport of branched-chain amino acids more highly expressed in the Bvg+ phase suggests that uptake of these amino acids is important in this phase. Therefore the RNAseq data point towards a Bvg+ phase in which genes coding for chaperones are more highly expressed suggesting proper folding of particular proteins is important, and genes coding for transport proteins suggesting that branched-chain amino acids, glutathione and glutamine are important for growth in this phase. On the other hand the genes more highly expressed in the Bvg- phase that are involved in cell division suggest that regulation of cell division may be different in this phase, while genes more highly expressed involved in the stress response suggest that this process is important for growth in the Bvg- phase.

The data fit with a model in which the Bvg+ is important in infection, and virulence genes are more highly expressed in this phase. Transport and iron scavenging genes upregulated in this phase would be expected to reflect the availability of nutrients during infection and the needs of the bacterium, for example siderophores to scavenge iron in a more iron-starved environment. On the other hand, the Bvg- phase might be expected to grow in a different environment, with a different availability of nutrients, hence why different genes for transporters are more highly expressed in this phase. Furthermore, it is interesting that stress responses and exporters would be more highly expressed in this phase and this suggests that the environment in which the Bvg- phase is growing is harsh, or conditions change frequently and the bacteria need to adapt.

5.1.4 The TCA cycle

One question that arose from the metabolic characterisation of growth of BP536 in the Bvg+ and Bvg- phases was whether the TCA cycle was more active in the Bvg+ phase. This came from observation that more glutamate is consumed in the Bvg+ phase to make less biomass, leading to the question of where the extra carbon being consumed is going. A hypothesis is that more carbon is lost as carbon dioxide in the Bvg+ phase during the TCA cycle. This would imply that there is more flux going through the TCA cycle in the Bvg+ phase than in the Bvg- phase, since this is where carbon dioxide is produced.

The RNAseq data provides information about whether genes that code for proteins involved in the TCA cycle are expressed more highly in either phase, which is useful in looking at the question of whether the TCA is more active in either phase.

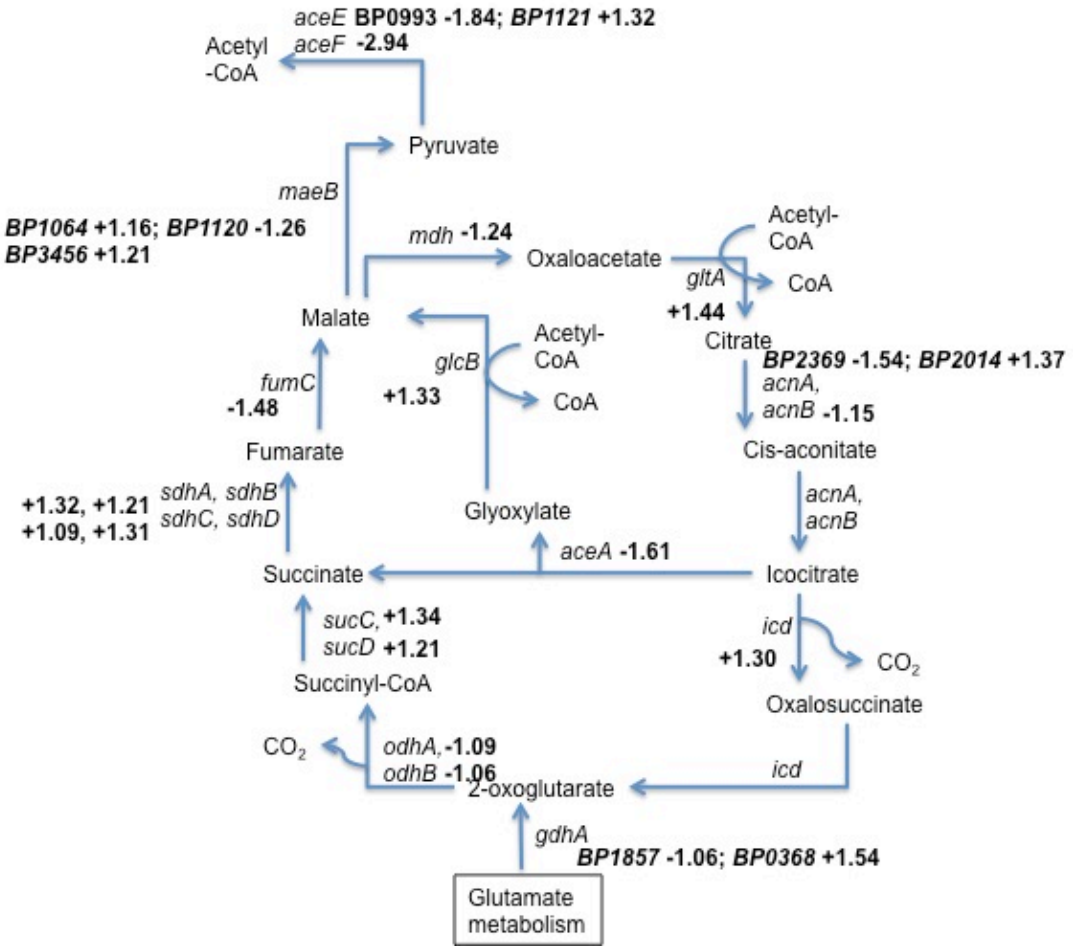


Figure 37- The TCA cycle of *B. pertussis* with expression differences. Genes for each pathway are annotated in italics. The phase in which genes are more highly expressed is denoted as either + or – next to a figure to denote the fold-change. Where more than one gene is annotated as performing the same function the gene numbers are given along with the fold-change for each gene.

The TCA cycle of *B. pertussis* with expression fold-changes is depicted in figure 37. The gene *aceF*, coding for a subunit of the pyruvate dehydrogenase, is the only gene that is greater than 2-fold more highly expressed in one phase over the other. An almost 3-fold expression change in *aceF* between the Bvg+ and Bvg- phases implies that there is more flux going though this pathway in the Bvg- phase. Indeed another subunit, *aceE* is also more highly expressed in this phase, albeit less than a 2-fold difference. An expression change of greater than 2-fold is defined as Bvg- regulated, but this is an arbitrary cut-off and it is possible that a change in expression of a gene of less than 2-fold could lead to more appreciable differences at enzyme level.

Looking at the expression of the TCA cycle as a whole it is not clear that it is more or less active in one Bvg phase or the other, although there are some differences. The higher expression of *gdhA* (*BP0368*) in the Bvg+ phase suggests more substrate

entering the TCA cycle in this phase The higher expression of *gltA* in the Bvg+ phase is interesting. The step catalysed by this gene product has been previously described as the rate-limiting step of the TCA cycle for *E. coli* under certain conditions where the TCA cycle is usually highly expressed [193], meaning that it is probably not necessary for the TCA cycle to be more highly expressed as a whole for there to be more flux through some pathways in one phase than the other.

There is a difference in the makeup of the cycle dependent on Bvg phase. Isocitrate can be used in two ways, to form 2-oxoglutarate in the two-step reaction catalysed by *icd*, or in the glyoxylate bypass, during which glyoxylate and succinate are formed, catalysed by *aceA*. The RNAseq data show that *icd* is more highly expressed in the Bvg+ phase, while *aceA* is more highly expressed in the Bvg- phase. This suggests that there is more flux through the part of the cycle from isocitrate to succinate, while there would be more flux through the glyoxylate bypass the Bvg- phase. This is interesting since the step catalysed by *icd* that produces carbon dioxide. The other step that produces carbon dioxide is the step catalysed by *odhAB*, which itself is not more highly expressed in one phase or the other, although it may be that more flux passes through this step due to the higher availability of 2-oxoglutarate from *icd* and *gdhA*, both more highly expressed in the Bvg+ phase. This would suggest more carbon dioxide is produced in the Bvg+ phase and could provide an explanation as to where extra carbon is going form the higher amount of glutamate being consumed per gram of biomass in the Bvg+ phase. As for why this would happen in the Bvg+ phase, the steps catalysed by these enzymes are also where NAD^+ is reduced to NADH to provide reducing power in the electron transport chain. This might suggest that the Bvg+ phase uses more NADH in the electron transport chain than the Bvg- phase.

5.1.5 Expression differences of other metabolic genes

There are at least 23 genes more highly expressed in the Bvg+ phase that are involved in metabolism, and 46 more highly expressed in the Bvg- phase in which it is the largest category of genes with 21% of genes more than 2-fold more highly expressed in the Bvg- phase involved in metabolism.

There are genes involved in the metabolism of fatty acids that are Bvg-regulated, with *fabD* and *fabG* more highly expressed in the Bvg+ phase and *BP0625* and *BP0219* more highly expressed in the Bvg- phase.

There are seven genes involved in amino acid biosynthesis that are Bvg-regulated; four are more highly expressed in the Bvg+ phase (*ilvC, ilvH, ilvI* and *his1*), and three are more highly expressed in the Bvg- phase (*soxD, amaB* and *ilvG*). In addition there are eight genes for enzymes that feed molecules into the TCA cycle that are Bvg regulated. These include *fahA*, *maiA* (*BP1955*) and lactate dehydrogenase (*lldD*), which are more highly expressed in the Bvg+ phase, and components of pyruvate dehydrogenase (*BP0628, pdhA, aceF*), as well as *maiA* (*BP0579*) and *pcaC*, which are more highly expressed in the Bvg- phase. *BP1955* and *BP0579* are both annotated as maleate cis-trans isomerases and are both named *maiA*, though the former is more highly expressed in the Bvg+ phase and the latter in the Bvg- phase.

Metabolic genes that are more highly expressed in the Bvg- phase code for a larger variety of functions. Eleven of these genes are involved in energy metabolism and include the Fe-S component of the cytochrome c reductase *petA* and components of the cytochrome bd complex *cydA* and *cydB*. On the other hand *atpC* and *atpD*, coding for components of the ATP synthase, are more highly expressed in the Bvg+ phase, as are the other components of this enzyme, although by less than 2-fold. There are also genes involved in amino sugars metabolism more highly expressed in the Bvg- phase, including *glmS*, which feeds glutamate into this pathway, as well as *glmU*, *bplA, bplD* and *bplB*.

The data show a wide and varied Bvg regulon that is involved in regulating a wide range of metabolic processes in *B. pertussis*. The higher expression of *ilv* genes in the Bvg+ phase and of pyruvate dehydrogenase in the Bvg- phase shows that pyruvate may be being used more to synthesise branched-chain amino acids in the Bvg+ phase and more to make acetyl-CoA in the Bvg- phase. Furthermore, the higher expression of *eno* in the Bvg- phase suggests a higher flux through gluconeogenesis pathways from pyruvate in this phase. This would suggest that synthesis of branched-chain amino acids is more important in the Bvg+ phase, while sugars would be synthesised from pyruvate more in the Bvg- phase. The difference in expression genes coding for enzymes of the electron transport chain is interesting, with NADH dehydrogenase being more expressed in the Bvg+ phase (possibly reflecting a greater

availability of NADH), and genes for the cytochrome bc_1 complex and the cytochrome bd complex more highly expressed in the Bvg- phase. The observation that genes coding for the ATP synthase complex are more highly expressed in the Bvg+ phase is interesting since it was shown that there is a greater PMF produced in this phase (Chapter 4). This would suggest that a greater amount of ATP is produced in this phase.

5.1.6 Grp

BP3008, annotated as *grp*, was 2.42-fold more highly expressed in the Bvg+ phase than in the Bvg- phase. Grp has been shown previously to play a role in regulating glutamate uptake in *E. coli,* where a cloned fragment from *Zymomonas mobilis* was introduced to an *E. coli* strain defective for glutamate transport. The clone showed increased glutamate uptake, although the product repressed expression of the H^+/glutamate transport system, GltP by interacting with the promoter of the *gltP* gene. This cloned gene was designated *grp* for Glutamate-uptake Regulatory Protein. Furthermore, a mutation in *lrp* in *E. coli* was complemented by expressing *grp*, leading to increase in expression of *ilvH,* a gene involved in biosynthesis of isoleucine and valine [194].

A *grp* knockout mutant was made to look at the effects on growth and glutamate consumption. Briefly, the primers listed in Chapter 2 were used in a PCR reaction to amplify regions either side of the *grp* gene. The process then proceeded in an identical fashion to that used to make the *petABC* mutant. The mutant was grown alongside BP536 in SS broth in Bvg+ and Bvg- phases in a 96-well plate growth assay.

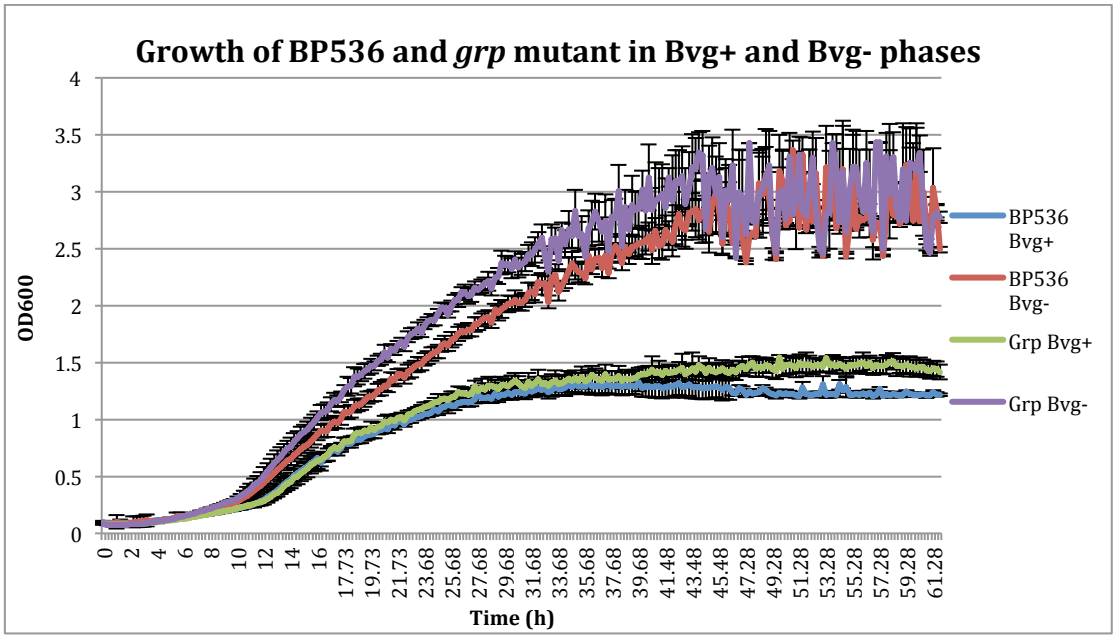


Figure 38– Growth of BP536 and the *grp* mutant grown in SS broth in the Bvg+ and Bvg- phases. The growth of BP536 and the *grp* mutant is shown over time as an increase in optical density. Growth was observed in a 96-well plate and measured until stationary phase. Data depicted is the average of 15 biological replicates, error shown is standard deviation.

There were fifteen cultures of BP536 and the *grp* mutant in both the Bvg+ and Bvg- phases. Three cultures from each condition were sacrificed during growth and supernatant harvested. Thus figure 38 shows the average growth of fifteen cultures at the beginning, the number of cultures diminishing as some are sacrificed to an average of three cultures at the end.

Figure 38 shows that the growth of the *grp* mutant has no growth defect in SS broth compared to BP536. Growth is similar between BP536 and the mutant.

The decrease in concentration of glutamate in the supernatant was measured between the start of growth at 0 hours and the end of exponential phase, when growth of the culture ceased, which corresponds best to the samples taken at 39.67 hours. Data is displayed in figure 39.

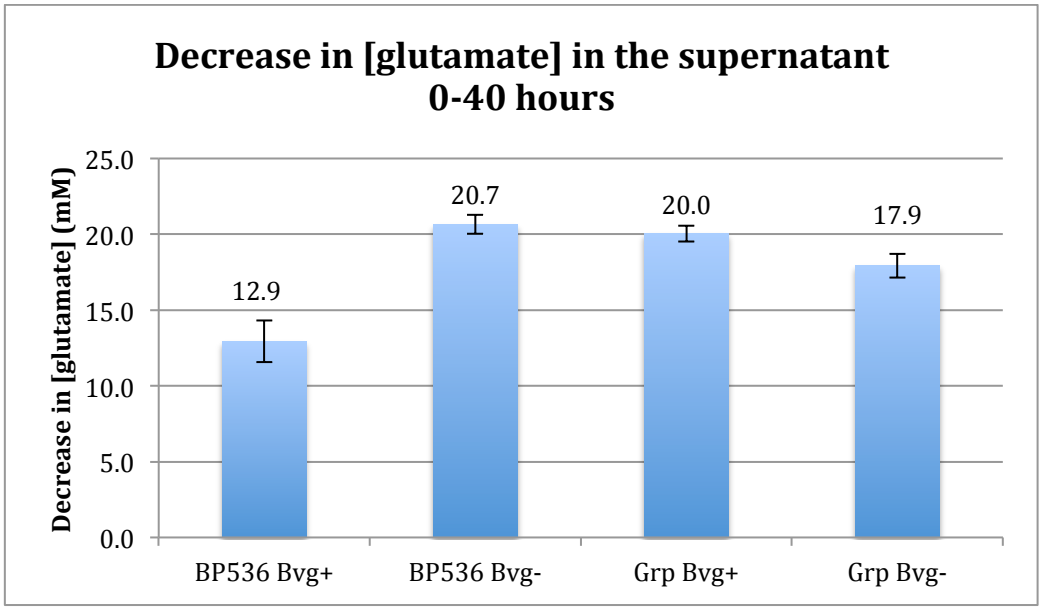


Figure 39– The average decrease in glutamate concentration in the supernatant between 0 and 37.67 hours. Glutamate concentration in the medium was measured at the beginning of the growth assay and again at 40h (taken to be the end of exponential phase). The net decrease in concentration of glutamate in mM was calculated and is depicated on the y axis.

The amount by which the glutamate concentration decreased in the medium was different between BP536 and the *grp* mutant in the Bvg+ phases (p=0.017). The amount of glutamate consumed from the medium is decreased in the *grp* mutant compared to BP536 in the Bvg- phase, though this difference is not significant (p=0.053).

During growth of the *grp* knockout, glutamate is consumed faster than in BP536 in the Bvg+ phase, providing evidence that during growth in the Bvg+ phase, when expression of *grp* is upregulated, the product of this gene plays a role in regulating the uptake of glutamate from the medium. This regulation appears to be in a manner that is repressive, since when this repression is relieved as in the *grp* knockout, growth is characterised by a significant increase in uptake of glutamate from the medium. This is despite the observation that growth is not improved in the *grp* mutant.

5.2 Discussion

5.2.1 The TCA cycle

Different parts of the TCA cycle are more active in one Bvg phase than in the other. There is more of a tendency for the Bvg+ phase to form glyoxylate from glycolate, while the Bvg- phase forms more glyoxylate (and succinate) from isocitrate, catalysed by *aceA*, which is more highly expressed in this phase, which might be expected leading to a greater flux through the glyoxylate bypass. On the other hand in the Bvg+ phase it would seem that there is increased succinate formation from isocitrate via 2-oxoglutarate and succinyl-CoA using the more highly expressed *icd* and *sucCD* genes. The *icd* gene, which does produce carbon dioxide is 1.3-fold more highly expressed in the Bvg+ phase, though it is not clear if this is enough to account for the greater consumption of glutamate per unit of biomass in the Bvg+ phase. Indeed the *odhAB* genes coding enzymes catalysing the conversion of 2-oxoglutarate to succinyl-CoA, also producing carbon dioxide is equally expressed in the two phases, though it may be that there is more flux through this part of the cycle in the Bvg+ phase since the gene coding for the *aceA* of the glyoxylate pathway is more highly expressed in the Bvg- phase.

Curiously genes that code for the enzymes in the pathways that involve the conversion of acetyl-CoA to CoA are more highly expressed in the Bvg+ phase (*gltA* and *glcB* with 1.44 and 1.33 fold-change in expression respectively). This implies a greater need for acetyl-CoA in the TCA cycle of *B. pertussis* in the Bvg+ phase.

Higher expression of a gene in one phase over another doesn't necessarily mean that it is directly regulated by Bvg as its expression could be controlled by another regulator, itself regulated by Bvg. One of the benefits of RNAseq is that it gives a picture of global expression meaning differences in expression between two conditions can be observed that are both direct and indirect results of growth in those conditions.

5.2.2 A different use of glutamate

There are several initial uses of glutamate. BP536 has two copies of *gdhA*, *BP0368* and *BP1857*, which convert glutamate to 2-oxoglutarate, a TCA cycle intermediate.

Alternatively *glnA* catalyses the converstion to glutamine and *glmS* feeds glutamine into the aminosugars metabolism pathways.

BP0368, one of the glutamate dehydrogenases is 1.54-fold more highly expressed in the Bvg+ phase, while *glmS* is 4.65-fold more highly expressed in the Bvg- phase suggesting glutamate is used differently between the two phases. The expression difference of *BP0368* is modest, but is suggestive of greater flux of glutamate through the TCA cycle, while the expression difference of *glmS* is much larger strongly suggesting more of a use for glutamate in amino sugars metabolism. It is probably no coincidence then that *eno*, coding for enolase, one of the first steps in gluconeogenesis, is 2.3-fold more highly expressed in the Bvg- phase as a product of this pathway is D-fructose-6P, which also feeds into aminosugars metabolic pathways. Furthermore, *glmU*, also involved in metabolism of amino sugars, is 3.04-fold more higly expressed in the Bvg- phase.

The RNAseq data suggest that there are different uses of glutamate depending on Bvg phase. *gdhA* is more highly expressed in the Bvg+ phase suggesting greater flux from glutamate through the TCA cycle, while *glmS* and *glmU* are more highly expressed in the Bvg- phase, suggesting greater flux from glutamate to amino sugars metabolism for peptidoglycan and other cell envelope component biogenesis. The observation that there are different uses for glutamate does not reflect the use of one pathway or the other, merely one being more active in one Bvg phase over the other. In a scenario in which the Bvg- phase is growing faster and dividing more often in might be expected that peptidoglycan would be made more quickly, hence the higher expression of genes synthesising precursors of peptidoglycan. Conversely the higher expression of *gdhA* (*BP0368*) in the Bvg+ could imply that there is greater flux through the TCA cycle due to more glutamate being channelled into this pathway.

5.2.3 A different use of pyruvate

The RNAseq data also shows an apparent difference in use of pyruvate. Pyruvate is generated from malate, a TCA cycle intermediate and then used as a precursor to many pathways including biosynthesis of amino acids and gluconeogenesis. Two genes coding for pyruvate dehydrogenase are more highly expressed in the Bvg- phase; *aceE* and *aceF*, which are 1.84 and 2.94-fold more expressed in this phase respectively. These genes catalyse the formation of acetyl-CoA from pyruvate. The

ilvBHI genes, however, are involved in the beginning of the biosynthetic pathways of valine, leucine and isoleucine from pyruvate and are 1.72, 2.12 and 2.63-fold more highly expressed in the Bvg+ phase respectively. This suggests that Bvg+ growth favours synthesis of amino acids from pyruvate, while Bvg- growth favours conversion to acetyl-CoA.

As suggested previously, the higher levels of β-HB seen in the Bvg- phase could be due a greater reliance on maintaining CoA levels, or a greater build-up of acetyl-CoA. This observation provides evidence for the latter scenario, particularly as the *fabG* and *fabD* genes are more highly expressed (2.3 and 3.15-fold) in the Bvg+ phase. These genes are involved in fatty acid synthesis and if this pathway were more active in the Bvg+ phase then it would require greater levels of CoA, a key part of the pathway.

Therefore it seems sensible to suggest that there is a greater build-up of acetyl-CoA in the Bvg- phase, due to the higher levels of pyruvate dehydrogenase as well as parts of the TCA cycle using acetyl-CoA being more highly expressed in the Bvg+. This may be the reason for the greater build-up of β-HB in the Bvg- phase, since CoA will still need to be recycled, and it would be by the condensation of two acetyl-CoA molecules to acetoacetyl-CoA and then conversion to PHB, later broken down to β-HB. However, *phbB*, the gene responsible for the production of PHB is 2.77-fold more highly expressed in the Bvg+ phase, and there are no genes responsible for conversion of acetyl-CoA to acetoacetyl-CoA that are clearly more highly expressed in the Bvg- phase. Again, it could be that more of a build-up of acetyl-CoA is enough for flux to increase through these pathways leading to a greater build-up of β-HB. There are two genes involved in the β-HB metabolic pathways, *BP3706* and *BP0217* which code for a putative enoyl-CoA hydratase and a 3-hydroxybutyryl-CoA dehydrogenase respectively, that are more highly expressed in the Bvg- phase (4.67 and 3.66-fold), but it is not clear precisely what role they play.

5.2.4 Energy metabolism

The RNAseq data reveals a difference in the make up of the electron transport chain depending on Bvg phase. The Bvg+ phase generates a greater PMF than the Bvg- phase (see Chapter 4), furthermore if the Bvg+ phase of *B. pertussis* has a more active

TCA cycle then it might be expected that more NADH would be available for oxidation by NADH dehydrogenase.

Indeed the RNAseq data shows that nine out of the fourteen genes coding for the NADH dehydrogenase complex are more than 1.3-fold more highly expressed in the Bvg+ phase, providing further evidence that more NADH is generated in the Bvg+ phase to provide reducing power to the electron transport chain. In addition five out of the eight genes coding for the ATPase are more than 1.5-fold more highly expressed in the Bvg+ phase, including two, which are more than 2-fold, more highly expressed. This may reflect a role for the larger PMF in the Bvg+ phase, and altogether suggests that the electron transport chain is more active in the Bvg+ phase to produce a greater PMF to feed a more highly expressed ATPase, which would be expected to produce more ATP.

On the other hand there are parts of the electron transport chain that are more highly expressed in the Bvg- phase. These include the *petABC* genes coding for the cytochrome bc_1 oxidoreductase, which are 2.53, 1.88 and 1.94-fold more highly expressed in the Bvg- phase. Also two components of the cytochrome c oxidase, *BP2171* and *BP2172,* are 1.82 and 1.86-fold more highly expressed in the Bvg- phase. It might be expected that these two components would be more highly expressed in the same phase since the former reduces cytochrome c and the latter oxidises it. Genes coding for the cytochrome bd complex are also more highly expressed in this phase (*cydAB,* 2.31 and 3.3-fold more highly expressed respectively). This cytochrome complex reduces oxygen and has been implicated in playing a role in oxygen limiting environments and in response to stress in other organisms [195, 196]. This may reflect the role of the Bvg- phase being environmental and coping with lower oxygen environments. The cytochrome bo complex, on the other hand, is encoded by four genes, *cyoABCD*, that are 1.35, 1.23, 1.05 and 1.41-fold more expressed in the Bvg+ phase respectively. This cytochrome complex has been implicated in playing a role in reducing oxygen in environments of oxygen abundance, perhaps reflecting the oxygen availability in the lungs in which *B. pertussis* is resident in the virulent Bvg+ phase [196].

Some of these fold-differences are only slight and it is not clear what phenotypic effect they have on the growth of *B. pertussis*. However, there is a clear difference in expression of genes coding for the electron transport chain dependent on Bvg phase, with the Bvg+ phase favouring use of the cytochrome bo complex, and the

Bvg- phase favouring the cytochrome bc_1 and bd complex. Without corresponding data showing protein levels or functional tests it is not possible to say if these expression differences translate into a difference in activity, however it was shown that there in the Bvg+ phase a greater PMF is generated (Chapter 4) showing that there is a difference at a functional level. Differences in electron transport chain may reflect the environments that the Bvg phases have evolved to cope with, where there is a more concentrated oxygen supply in the lungs, and the possibility of oxygen restriction in the environment. *B. pertussis*, however is a human restricted pathogen with no environmental reservoir, so this difference may have evolved to suit a common ancestor of *Bordetella spp.* which would have had an environmental phase of life [1].

It is not clear what impact the differences in makeup of components of the electron transport would have on PMF generated. It is clear that a larger PMF is generated in the Bvg+ phase and this would be expected to be a consequence of differential expression of part of the electron transport chain. A contributor to this would be a more highly expressed NADH dehydrogenase, which would be able to oxidise more NADH produced in the TCA cycle. Two genes annotated as formate dehydrogenase (*BP1513* and *fdhC*) are more than two-fold more highly expressed in the Bvg+ phase. *fdhC* is in an operon with *fdhA* and *fdhB,* which have 1.87 and 2.24-fold greater expression in *B. pertussis* respectively, however *fdhB* is a psuedogene in BP536, so it is not clear if a working formate dehydrogenase is expressed. Theoretically, the protein couples the oxidation of formate with the production of NADH and could reflect a greater amount of NADH produced by the Bvg+, showing even if not expressed, the greater tendency to form NADH in the Bvg phase even if this particular process does not take place in this strain or even this species. Furthermore the higher level of expression of the ATP synthase provides evidence that the higher PMF generated is to generate more ATP. This may reflect the greater need for ATP in the Bvg+ phase to work transport systems to secrete virulence factors such as adhesins and toxins outside of the cell.

5.2.5 Grp- a regulator of glutamate uptake in *B. pertussis*

The *grp* gene was chosen for further investigation because it is annotated as Glutamate-uptake Regulatory Protein and is 2.42-fold upregulated in the Bvg+ phase.

Grp regulates glutamate uptake in *Z. mobilis* [194]. This is an interesting observation since the major component of most media used to grow *B. pertussis* is glutamate.

The gene was shown to negatively effect the expression of GtlP, a H^+-coupled transporter of glutamate. The genome of *B. pertussis* does not contain a recognised H^+/glutamate transporter. Of the annotated glutamate transport systems in *B. pertussis* there are two copies of a *gltJKL* permease and one gene, *gltS*, coding for a Na^+/glutamate symporter. None of these genes are identified as Bvg-regulated by RNAseq, although the *gltJKL* genes (*BP0054-0056*) are 1.33, 1.49 and 1.17-fold more upregulated respectively in the Bvg+ phase. Furthermore, *ilvH,* shown to be positively regulated by Grp in *Z. mobilis [194]*, is 2.63-fold upregulated in the Bvg+ phase while the genes immediately upstream and downstream, *ilvI* and *ilvC*, are 2.12-fold and 3.07-fold more expressed in the Bvg+ phase respectively.

A *grp* mutant of *B. pertussis* displayed no growth defect in SS broth. The observations from glutamate uptake from the medium show that for BP536 in the Bvg- phase more glutamate is taken up between 0 and 39.67 hours of growth, which is expected as it is growing faster. In the mutant, however, there is no difference between the amount of glutamate taken up from the medium between growth in the Bvg+ and Bvg- phases, both of which take up significantly more glutamate from the medium than BP536 in the Bvg+ phase. This provides evidence for *grp* playing a role in regulating glutamate uptake, since the mutant lacking *grp* behaves in the Bvg+ phase like BP536 in the Bvg- phase where *grp* is present but expression is downregulated.

How *grp* regulates glutamate uptake in *B. pertussis* is not clear, although regulation would appear to be negative since the mutant takes up more glutamate from the medium than BP536. Grp probably acts as a transcription factor, since it was found to bind the promoter of *gltP* in *E. coli* [194], in which case it would be expected that effects on the expression of genes by *grp* would be seen in the RNAseq data, where *grp* is more highly expressed in one Bvg phase than in the other. *B. pertussis* does not express *gltP*, and of the recognised glutamate transport genes there do not appear to be any that are negatively regulated by expression of *grp* (i.e. more highly expressed in the Bvg- phase). Therefore it is clear that *grp* negatively effects glutamate uptake in *B. pertussis*, but it is not clear how. It remains to be seen whether there are other glutamate transport systems expressed by *B. pertussis* that are more highly expressed in the Bvg- phase.

The *ilvHI* operon is involved in biosynthesis of isoleucine, leucine and valine. It has been shown to be positively regulated by *grp* in *E. coli* [194], and is more highly expressed in the Bvg+ phase of BP536 during which *grp* is also more highly expressed. It is not clear whether expression of *grp* directly effects expression of the *ilvHI* operon or whether expression is due to other factors in *B. pertussis*, but regardless the picture is of Bvg+ phase growth repressing glutamate uptake and activating synthesis of branched-chain amino acids.

Chapter 6- Conclusions

6.1 Growth dynamics are dependent on Bvg phase

The Bvg- phase has been recognised as having a growth advantage over the Bvg+ phase, for example spontaneous Bvg- mutants can outcompete Bvg+ bacteria in vaccine culture [144]. However, this report is the first to characterise the differences in growth between *B. pertussis* grown in the Bvg+ and Bvg- phase. From growing BP536 in the Bvg+ and Bvg- phases side-by-side in a 96-well plate it is clear that in the Bvg- phase bacteria reach a higher cell density than in the Bvg+ phase in SS broth. By looking at the growth dynamics and calculating growth rates and doubling times it is clear that this higher yield is reached by a combination of exiting lag phase sooner than in the Bvg+ phase and maintaining significantly higher growth rates during early exponential phase growth. Growth is slowed despite glutamate levels still being in the millimolar range. This is probably due to autoinhibitory molecules being produced during growth affecting growth of *B. pertussis* regardless of Bvg phase

Maintaining higher growth rates means that cells in the Bvg- phase are dividing more often. This means one of two things; either *B. pertussis* cells reach a uniform length or grow by a uniform amount regardless of Bvg phase which would mean Bvg- phase cells would have to grow (elongate) faster, or cells in the Bvg- phase are smaller and reach a smaller threshold of size of growth before dividing. CFU data show that there are the same amount of cells per ml of culture at a given OD in the Bvg+ phase as in the Bvg- phase, and biomass data suggest that 1ml of culture for a given OD has the same mass in the Bvg+ phase as in the Bvg- phase. This suggests that the former scenario is the likeliest since the same number of cells have the same mass regardless of Bvg phase, so they are likely to be of a similar size.

If this is the case then clearly cells in the Bvg- phase have to elongate quicker before dividing. Further evidence to this is the conditional essentiality of *mreB*. Disrupting the normal process of cell wall elongation by mutation is lethal in the Bvg- phase but not in the Bvg+, demonstrating that the process of cell elongation is critical for Bvg- phase growth. The reason why disruption of this process is not lethal for growth in the Bvg+ phase is not clear, but it may be that since cell division happens less frequently that PBPs other than *mrdA* are sufficient to synthesise enough peptidoglycan to maintain cell growth and division as spheres.

Faster elongation of cells in the Bvg- phase would require faster synthesis of peptidoglycan. This is demonstrated by the higher level of expression of genes coding for enzymes involved in gluconeogenesis and aminosugars synthesis pathways, which lead to the synthesis of precursors of peptidoglycan.

6.2 Metabolism is dependent on Bvg phase

It is not just cell growth and division that are affected by Bvg phase, different metabolic processes are also more active in different Bvg phases. This is inferred by the findings that more β-HB is secreted into the medium in the Bvg- than in the Bvg+ phase and that more glutamate is consumed to make 1g of biomass in the Bvg+ phase than in the Bvg- phase.

Clearly if cells in the Bvg+ phase are consuming more glutamate to make biomass then extra carbon is being used in a way that is not accounted for in biomass and it was postulated that it could be released at carbon dioxide produced during metabolism, specifically by the TCA cycle. The RNAseq data is useful in providing information about metabolic reactions through which more flux may pass, although this assumes that transcriptomic data always corresponds to levels of protein and that this corresponds to flux through a reaction, which is a simplistic way of viewing metabolism. From RNAseq data it is not clear that the TCA cycle as a whole is more active in the Bvg+ phase, however there is evidence that pyruvate dehydrogenase is more active in the Bvg- phase since the components *aceF* and *aceE* are nearly 3 and 2-fold more highly expressed respectively, suggesting that the reaction catalysed by this enzyme is more active in the Bvg- phase. Furthermore, from RNAseq data there might be expected to be a greater flux through the glyoxylate pathway in the Bvg- phase. Conversely, the higher level of expression of *icd* in the Bvg+ phase might be expected to lead to a greater flux through the part of the cycle from isocitrate to 2-oxoglutarate and succinyl-CoA in this phase, which is the part of the cycle during which carbon dioxide is produced. In summary although there are differences in the activity of the TCA cycle at a transcript level dependent on Bvg, though it is not clear how this might lead to changes at the level of protein or enzyme activity.

Data from the TraDIS led to the investigation of a *petABC* mutant, which led to the observation that there is a greater PMF produced by the Bvg+ phase than the Bvg- phase. It is clear from the RNAseq data that when looking at the electron

transport chain as a whole, there are different components that are more highly expressed in different Bvg phases. This includes a difference in expression in the complexes for reducing oxygen as the last step in the chain whether the cytochrome bo, bd or c complexes. This probably reflects the environment in which Bvg would be active or not active, i.e. high abundance of oxygen in the lungs and potential low abundance in other environments. A majority of components of the NADH dehydrogenase are more than 1.3-fold more highly expressed in the Bvg+ phase. This could be because there is more NADH produced by more active components of the TCA cycle that are more highly expressed in this phase. Furthermore, the ATP synthase is more highly expressed in the Bvg+ phase, which would be the end result of a more active TCA cycle, producing more reducing power and a higher PMF. This would be expected to produce more ATP, although the reasons for this are unclear.

In summary there are major differences in the metabolism of *B. pertussis* depending on Bvg phase. This includes the Bvg- phase to using more glutamate to synthesise aminosugars, while the Bvg+ phase channels more glutamate into the TCA cycle. The Bvg+ phase would use more pyruvate in reactions to synthesise branched-chain amino acids, while the Bvg- phase channels more into the gluconeogenesis pathway and to make acetyl-CoA, leading to higher levels of β-HB measured during growth assays Furthermore, differences in expression of genes coding for enzymes involved in the TCA cycle and electron transport chain suggest increased production of carbon dioxide, NADH and ATP in the Bvg+ phase.

It should be noted that the differences seen in the expression of genes dependent on activity of the Bvg system have not all been shown to be directly related to the activity of Bvg. There exists the possibility of a complexity of signalling that goes beyond Bvg, including transcriptional regulation by secondary regulators and it should be noted that there are many genes for putative transcriptional regulators that are upregulated depending on Bvg activity. Furthermore, all experiments were performed using 50mM $MgSO_4$ to modulate cultures to the Bvg- phase, therefore it is not known whether changes in expression of genes seen are due to Bvg activity or another factor such as osmolarity of the medium. Modulation was used as it represents Bvg activity switching on and off in response to a ligand as would be expected to happen in the environment (though it is not known to what ligand Bvg responds *in vivo*). It would be interesting to look at growth characteristics and transcriptomics for a *bvg* mutant to see how similar those data would be to those seen

here with modulation. It should be noted however, that there are patterns of gene regulation by Bvg in the RNAseq data presented here that are expected, such as high expression of virulence factors in the Bvg+ phase and gene for the capsule in the Bvg- phase, which do validate the data. Furthermore, differences in expression of metabolic genes dependent on Bvg activity have been described previously for *B. bronchiseptica* [143] and while one should compare data from two different species with caution, *B.pertussis* did evolve recently from a bronchiseptica-like ancestor [1], allowing for a certain degree of validation between data sets where similarities are seen. In any case, the wide range of functions assigned to genes whose expression is regulated (directly or indirectly) by conditions under which activity of Bvg changes uncover the true regulatory potential of the Bvg two-component system which in part can begin to explain differences seen with regard to growth phenotype.

6.3 Using the Bvg- phase as a vaccine platform

One of the purposes of studying the metabolism of the Bvg- phase of *B. pertussis* is because potentially this phase has a role in vaccine production. Cultures in the Bvg- phase grow to 1.7-times the cell density of cultures in the Bvg+ phase, meaning that if they could be engineered to express virulence factors then this could improve the yield of vaccine antigens.

However, it may not just be a case of expressing these vaccine antigens in the Bvg- phase. The physiology of Bvg- phase growth is fundamentally different to Bvg+ phase growth, which may be slower and produce lower yields because its metabolism is refined because it needs to produce these virulence factors. In other words the makeup of the Bvg regulon probably reflects the environments in which the Bvg phase is active or not, and any metabolic pathways affected by changes to Bvg activity could be linked to the necessity, or not, of expressing virulence factors. The metabolism of the Bvg+ phase may have evolved to deal with synthesising and secreting virulence factors, which may mean that if *B. pertussis* were engineered to express these factors they may not do so as readily or in the same abundance as in the Bvg+ phase. Alternatively, expressing virulence factors in the Bvg+ phase may be a metabolic burden, leading to an overactive TCA cycle losing carbon as CO_2. In this case it may be that the Bvg- phase would be a good model to express virulence

factors, providing they don't have a knock-on effect on metabolic process, skewing them to become more like those of the Bvg+ phase.

On the other hand the Bvg+ phase could be engineered to have a metabolism more similar to the Bvg- phase. This could be done by taking key metabolic genes expressed more highly in the Bvg- phase out of Bvg control and expressing them constitutively, genes such as *glmS*, *aceEF*, *petABC*. However, it is possible that simply changing the expression of one gene may not be enough to radically change the growth phenotype of the Bvg+ phase, and it may be necessary to change the expression of a number of genes at once to drive flux through a particular pathway.

This study has provided the first characterisation of the growth phenotype and metabolism of the Bvg- phase of *B. pertussis*. Functional genomics-based approaches such as TraDIS and RNAseq have been used in combination with assaying mutants to produce a picture of how the metabolism is different between the Bvg phases and why there is a different growth phenotype in culture dependent on Bvg phase. This work may be instrumental in the future in providing a rationale for designing a strain of *B. pertussis* that would have a Bvg- growth phenotype but express virulence factors. Such a strain could be valuable to companies looking to improve antigen yields of culture for producing the acellular vaccine.

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